THE STRUCTURE OF THE INSECT EGG-SHELL IN RELATION TO THE RESPIRATION OF THE EMBRYO

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(With Two Text-figures)

Many insect eggs are able to avoid mechanical injury and desiccation because they are protected by an egg-shell or chorion which is tough, rigid and relatively waterproof. These properties tend to make it impermeable to gases, and since the egg contains a living embryo, there must be provision for the diffusion of an adequate volume of oxygen into the egg. The way in which this is achieved has never been satisfactorily explained. Specialized respiratory structures have been described in many species of insect egg, especially in those of parasites (Maple, 1937), and a respiratory function has sometimes been ascribed to the system of micropylar canals that penetrate the shell in hard-shell eggs, but until now there has been no direct evidence for the respiratory function of these structures. Indeed it seems unlikely, at first sight, that sufficient oxygen could diffuse through such small areas of the shell to satisfy the needs of the embryo, although in some species there can be little doubt that oxygen uptake is restricted to a small part of the shell; in certain species of capsid, for instance, the eggs are embedded in the tissues of a plant with only the caps exposed to the air.

What follows is an account of an investigation of this problem in the eggs of the reduviid bug, *Rhodnius prolixus* (Stahl). Beament (1947) has shown that the egg-shell in this species is composed of several layers, and its waterproofing properties are due to a thin continuous layer of wax on its inside surface. He has also shown that at the cap end of the egg the shell is penetrated by a series of micropyles and pseudomicropyles. I have been able to show that most of the oxygen consumed by the egg enters through the micropyles and pseudomicropyles, and that the relative impermeability of the rest of the shell is due, not to the wax layer, but to the components that give the shell its mechanical strength. From the theoretical point of view it can be shown that sufficient oxygen can only enter the egg through these pores if there is a continuous gas phase under the shell. A gas space can be demonstrated in the *Rhodnius* egg, and its minimum dimensions can be calculated.

EXPERIMENTS TO DETERMINE THE SITE OF OXYGEN UPTAKE

The oxygen consumption of groups of eggs was measured before and after selected areas of the shells had been covered with wax. To ensure that the effects were due to the smothering action and not to the toxic properties of the wax, control experiments were carried out using shellac, vaseline and gelatine. Eggs of different ages were studied, Beament having shown that the composition of the chorion changes during development.

Experimental procedure. In each experiment three groups of eggs of the same age (maximum variation 24 hr.) incubated at 25° C. were used, 70 % R.H. The number of eggs in a group varied in different experiments and is recorded in the table of results (Table 1).

Table 1. *Results of smothering experiments*

No. of exp.	Age of eggs (days)	No. of eggs	mm.^{3} O_2/egg/hr.		Treatment
			Untreated	Treated	
78 i	3–4	6	0·082	0·018	Cap end covered with low m.p. wax
ii		4	0·092	0·020	Cap end covered with low m.p. wax
iii		6	0·080	0·088	Control untreated
79		6	0·088	0·020	Posterior end covered with low m.p. wax
81 i	7	3	0·101	0·011	Cap end covered with low m.p. wax
ii		3	0·106	0·014	Cap end covered with low m.p. wax
iii		3	0·107	0·021	Cap end covered with low m.p. wax
97 i	10	2	0·122	0·014	Cap end covered with vaseline
ii		2	0·102	0·026	Cap end covered with vaseline
90 i		3	0·181	0·028	Cap end covered with wax
ii		3	0·152	0·020	Cap end covered with wax
iii		3	0·145	0·025	Cap end covered with wax
83 i		4	0·150	0·020	Cap end covered with gelatine
ii		4	0·136	0·018	—
iii		4	0·117	0·021	—
84 i	11	4	0·134	0·115	Posterior end covered with wax
ii		4	0·164	0·158	—
iii		4	0·131	0·122	—
82 i	12	4	0·108	0·012	Cap covered with shellac
ii		4	0·143	0·010	—

The oxygen consumption of the eggs was measured at 25° C. by means of a new type of microrespirometer which is to be described elsewhere, capable of measuring the rate to the nearest 0·001 mm.^{3}. The rate was recorded over a period of 1 hr., then the eggs were removed from the respirometers and a selected area of the shell covered with wax (m.p. 30° C.) by means of an electrically heated microcautery. The eggs were gripped in a pair of fine forceps controlled by a screw clamp. A drop of wax, held on the wire loop of the cautery, was brought up to the egg and heated until it just melted. It was then touched against the egg and quickly withdrawn. The wax adhering to the egg solidified immediately. To ensure that the seal was complete the edges of the wax drop were carefully remelted and again allowed to solidify.

After treatment, the groups of eggs were returned to the same respirometers from which they had been removed, and the oxygen consumption measurement was repeated.

In the control experiments the vaseline was placed on the egg in the same way as the wax, the shellac was put on with a camel hair brush and the gelatine covers by holding the eggs in gelatine solution until it solidified, and then cutting out the block containing the egg with a sharp scalpel.

Results. The experimental results are shown in Table 1. It will be seen that whatever compound was used for covering the egg, the results were essentially the same. The low melting-point wax was, however, the more convenient material and was used in most of the experiments.

Blocking the cap end of the egg reduces the oxygen consumption to a value of about 0·02 mm.^{3}/egg/hr., irrespective of the initial rate or age of the eggs, whereas if the posterior end was covered the rate of oxygen consumption was not affected (Exp. 84). There was an exception, however; young eggs, less than 5 days old, show a reduction in rate if the posterior end of the egg is covered with wax (Exp. 79). These young eggs are delicate and the reduction in the respiratory rate is probably the result of injuring the egg and not to the suffocating effect of the wax. It was found that this treatment caused the death of the 3-day-old eggs, but waxing the posterior end of the older eggs had no effect on hatching. The remarks which follow refer to eggs which are more than 6 days old.

It was clear from these results that although oxygen is absorbed through the whole surface of the shell, most of it enters through the cap end. The value obtained for the rate at which the gas is absorbed by eggs in which the cap has been covered by wax probably represents the limiting rate of oxygen diffusion through the egg membrane at the partial pressure of oxygen in the atmosphere.

It is interesting that this value does not change appreciably as the egg gets older. The additional wax which, according to Beament (1946), is laid down during development does not seem to affect the permeability to oxygen.

Table 2. *Results of smothering experiments, in which different parts of the cap were covered with wax*

No. of exp.	Age of eggs (days)	No. of eggs	mm.^{3} O_2/egg/hr.		Treatment
			Untreated	Treated	
115	11	3	0·149	0·099	Centre of cap covered, rim partially covered
116	12	3	0·145	0·144	Centre of cap covered, rim clear
117	12	3	0·154	0·157	Centre of cap covered, rim clear

In the foregoing experiments the wax cover enveloped the whole of the anterior part of the egg. A further set of experiments was therefore carried out, in which the effect of covering the centre of the cap was compared with the effect of covering the rim (Exps. nos. 115, 116, 117, Table 2). The results of these experiments show clearly that it is in the region of the rim that oxygen passes into the egg.

Beament has shown that round the rim of the cap there are two series of small canals passing through the egg-shell; the pseudomicropyles and the true micropyles. The position of these structures is shown in Figs. 1 and 2.

It will be seen that the pseudomicropyles are closed at both ends except for minute pores at the outer end which penetrate the exochorion. At the inner end, the pseudomicropyles do not penetrate the inner wax layer, but end a short distance from it. There are some 200 of these structures and the number is approximately constant in eggs of all ages (Beament, 1947).

The true micropyles are much larger and they are open at the outside end, but like the pseudomicropyles they do not penetrate the inner wax layer. They are few in number, averaging about 13 per egg, and, moreover, the number may be considerably reduced in eggs laid by older females. The oxygen consumption of eggs

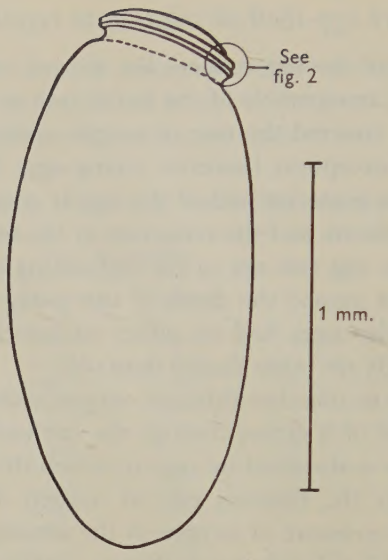

Fig. 1. The egg of *Rhodnius prolixus*.

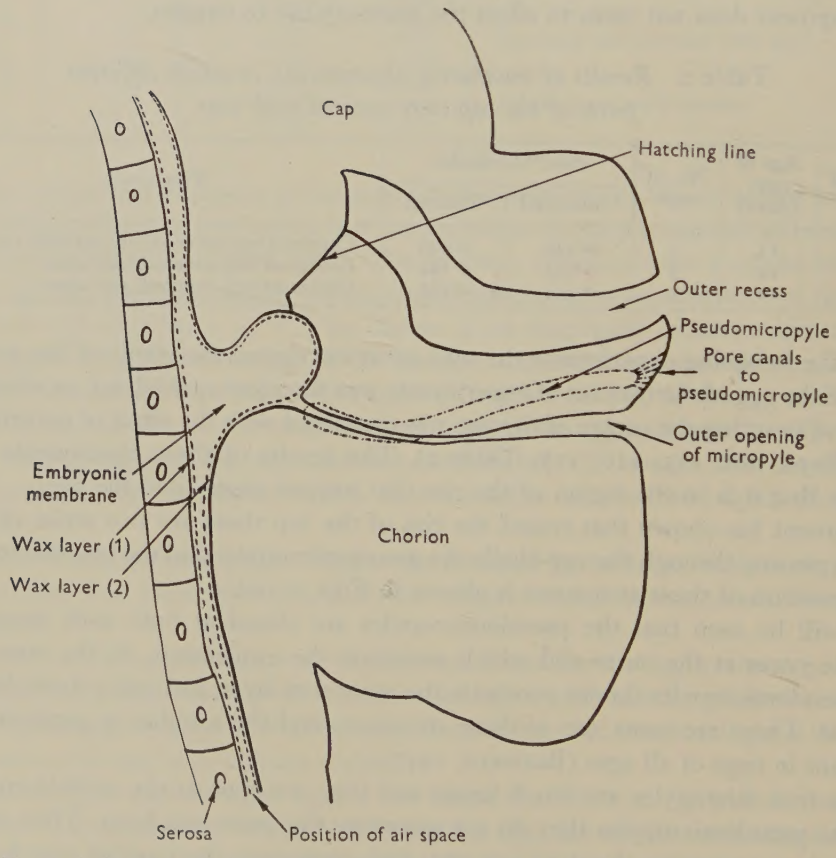

Fig. 2. A diagram of a longitudinal section through the part of the shell indicated by the circle in Fig. 1 (after Beament).

laid by old females does not differ appreciably from that of eggs laid by younger ones. This suggests that it is the pseudomicropyles that are the main site of gaseous diffusion into the egg.

THE DIFFUSION OF OXYGEN INTO THE EGG

It is difficult to see how an adequate supply of oxygen can reach the embryo through such a small area of the egg surface. No precise analysis of this problem is possible because the shape of the egg and the canals are such that the mathematical treatment would be too complicated. But it is possible to consider a simplified system, to which Hills's equations (Hill, 1928) for the penetration of oxygen into respiring tissues can be applied, which gives some idea of the problem and its solution.

Consider a sheet of tissue consuming oxygen at the same rate per unit volume as the *Rhodnius* egg; then assuming a concentration of oxygen at the free surface equal to that in air, and assuming a diffusion coefficient for the tissues it is possible to calculate the depth at which the oxygen concentration is zero. Thus

$$\text{the depth } b' = \sqrt{\frac{2ky_0}{a}},$$

where k = diffusion coefficient, y_0 = partial pressure of oxygen at surface, a = rate of oxygen consumption in c.c. of O_2/min.

Let $k = 1{\cdot}48 \times 10^{-5}$ (i.e. k for muscle at 20° C.), $y_0 = 0{\cdot}21$ atm., $a = 3{\cdot}47 \times 10^{-3}$ (rate on sixth day); then $b' = 0{\cdot}042$ cm.

Similarly, if the diffusion coefficient for water is substituted for that of muscle, the value of b' is 0·064 cm.

The egg is 0·17 cm. long and so there would be no oxygen available more than half way down the egg even if the cap were removed and the end of the yolk exposed to the air.

This treatment assumes that the whole of the egg contents consume oxygen uniformly throughout. If we assume that the yolk does not consume oxygen, which is unlikely, and that the embryo is consuming oxygen at 1×10^{-4} c.c./hr. at a point 0·1 cm. from the cap, that is about half-way down the egg, then we can calculate the apparent permeability coefficient (P) of oxygen through the yolk. Thus

$$\frac{dm}{dt} = P_{\text{yolk}} A \frac{\Delta p}{\Delta x},$$

$$\text{area of cap} = \pi \times 9 \times 10^{-4} \text{ cm.}^2,$$

$$\Delta p_{O_2} = 0{\cdot}21 \text{ atm.},$$

$$\frac{dm}{dt} = \frac{1 \times 10^{-4}}{3600} = 2{\cdot}78 \times 10^{-8},$$

$$\Delta x = 0{\cdot}1 \text{ cm.},$$

therefore

$$P^{O_2}_{\text{yolk}} = \frac{dm}{dt} \frac{\Delta x}{\Delta p A} = \frac{2{\cdot}78 \times 10^{-8} \times 0{\cdot}1}{0{\cdot}21 \times \pi \times 9 \times 10^{-4}} = 4{\cdot}6 \times 10^{-6}.$$

Thus the permeability coefficient of the yolk would appear to be some ten times greater than that for water $P^{O_2}_{water} = 5{\cdot}8 \times 10^{-7}$.

There is no data for the permeability of oily solutions to O_2, and in view of the greater solubility of O_2 in oils it might be thought that, as the yolk contains oil, a greater permeability coefficient would be expected. But it must be remembered that the case considered here is that of an egg with the cap removed and the surface of the yolk exposed to the air, whereas in the normal egg the air enters through a very small pore round the cap.

If, however, we assume that the yolk is exposed to air on all sides, Hills's equation for the limiting thickness of a cylinder of respiring tissue can be applied (Hill, 1928, equation 25). Substituting the same constants as before in this equation:

$$r_0 = \sqrt{\frac{4k_0}{a}}$$

$$= \sqrt{\frac{4 \times 1{\cdot}47 \times 10^{-5} \times 0{\cdot}21}{3{\cdot}4 \times 10^{-3}}}$$

$$= 0{\cdot}060 \text{ cm.}$$

The radius of the *Rhodnius* egg is 0·03 cm., which is well within the limit for the complete permeation of a cylinder of tissue respiring at this rate in air.

At first all attempts to demonstrate a layer of air inside the shell were unsuccessful. Small bubbles of air could sometimes be seen when the eggs were squashed under water, but it was not easy to see where they came from. Eggs were also placed in a container of boiled water and subjected to a low pressure, when it was found that bubbles, large enough to make the eggs rise in the water, appeared round the rim of the cap. These bubbles might have been due to air trapped in the groove round the rim, although the eggs had previously been wetted with alcohol. These experiments showed that even if the air was present under the shell it was in very small amounts.

An air space under the shell was eventually demonstrated by illuminating an egg with strong transmitted light under a binocular microscope, and observing the changes in the appearance of the shell when drops of odourless kerosene were placed, first on the cap rim and then on the shell. In both cases the spreading of the oil can easily be seen because it decreases the light scattered by the shell. When the oil is placed on the outside it spreads unevenly in all directions and evaporates, but when it is placed on the rim of the cap it immediately encircles the egg and the smooth boundary of the oil can be seen moving towards the tail end of the egg. It is clear that in this case the oil is passing down under the shell, because it does not evaporate, and the two boundaries can be seen to pass each other if drops are placed simultaneously on the cap rim and on the surface of the shell. It only takes 4 sec. at room temperature for the oil to reach a point half-way along the egg so that there is little doubt that it is moving through a gas-filled space between the shell and the serosa. An attempt is now being made to determine the dimension of this space, which is probably very small. A space only 1×10^{-5} cm. width, however,

is sufficient to provide a diffusion path wide enough to ensure an adequate supply of O_2 to the whole of the egg surface.

There must be some means of preventing this gas space from collapsing, therefore it will probably be found to consist of a labyrinth of small interconnected pores, rather than a continuous space. Furthermore, if these pores occur in, or are lined with, the waxy substance that Beament has found under the chorion, then they would be able to prevent the invasion of the gas space by water. This is an important factor because the life of the embryo depends on the gas space being uninterrupted.

OXYGEN CONSUMPTION AND WATER LOSS IN THE *RHODNIUS* EGG

Having described the relationship between the structure of the egg-shell and the respiration of the egg, it is now possible to consider the problem of how the waterproofing properties of the shell are reconciled with the need for an adequate supply of oxygen.

Beament has shown that the waterproofing properties of the *Rhodnius* egg are due to a thin layer of wax laid down on the inside of the shell (Beament, 1946). But although this enables the egg to develop in atmospheres of low relative humidity, it does not prevent some loss of water. He reports that at 0 % R.H. the egg loses 0·09 mg. water per cm.^{2} of surface per hr.; this represents the passage of some 3 mm.^{3} of water vapour per hr. through the egg-shell. It will be recalled that the maximum rate at which oxygen will diffuse through the general surface of the egg at atmospheric pressure is about 0·02 mm.^{3}/hr. This relatively low permeability to oxygen cannot, however, be due to the wax layer since this is continuous over the whole of the shell and is not penetrated by the micropylar canals. The wax must in fact be very permeable to oxygen since it has been shown experimentally that more than 0·2 mm.^{3}/hr. can pass into the egg through a very small area of wax at the ends of the pseudomicropyles. Therefore the impermeability to oxygen must be due to other components of the shell, and it would seem that an adequate waterproofing mechanism is in no way incompatible with the absorption of a sufficient supply of oxygen, because the *Rhodnius* egg can survive the loss of several mm.^{3} of water vapour per hr. but absorbs not more than 0·3 mm.^{3} of oxygen per hr. It is probably the component of the shell that gives strength rather than its waterproofing properties that restricts the diffusion of oxygen. The two are, however, not completely unrelated, for as Beament points out, the waterproofing wax has to have a high melting-point if it is to be efficient at high temperatures; such wax layers are probably brittle, and require a mechanical support (Beament, 1946). Thus the thick rigid egg-shell of the *Rhodnius* egg fulfils a very important function by providing a firm support for the wax layer, and as the sites at which oxygen is able to enter the egg are restricted to small pores in the egg-shell, only small areas of the wax layer are unsupported.

The *Rhodnius* egg thus maintains a cleidoic habit in conditions of high temperatures and low humidities, by reason of its tough rigid shell which supports a waterproof layer of high melting-point wax on its inner surface. The mechanical component of the shell restricts the uptake of oxygen through the general surface, but

oxygen is able to reach the embryo through a series of small pores in the rim of the cap—the micropyles and pseudomicropyles. These pores communicate with a gas space under the chorion which ensures that all parts of the egg can obtain an adequate supply of oxygen.

SUMMARY

1. The site of gaseous exchange in the eggs of *Rhodnius prolixus* (Stahl) is shown to be the rim of the cap which covers the anterior end of the egg. Most of the oxygen consumed by the embryo enters the egg through the micropyles and pseudomicropyles which penetrate the shell in this region.

2. The physical conditions necessary for the passage of sufficient oxygen through these pores is discussed. A continuous gas space under the shell—the presence of which can be deduced on theoretical grounds—is shown to exist in the *Rhodnius* egg.

3. The relationship between waterproofing and the permeability of the shell to oxygen is discussed.

My thanks are due to members of the Zoology Department who have helped in various ways during the course of this work and especially to Dr Thorpe and Dr Crisp for helpful criticism and suggestions, to Dr Townsend of the Cavendish Laboratory for help with the physical problems involved in interpreting the results.

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ON THE REACTIONS TO HUMIDITY OF *PERIPATOPSIS MOSELEYI* (WOOD-MASON)

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(With Seven Text-figures)

Quantitative studies have been made by previous workers on the reactions to humidity of three classes of terrestrial arthropod. The isopods have been studied by Gunn (1937) and Waloff (1941), the sheep tick by Lees (1948), while a number of insects have been examined in this connexion. The Onycophora have so far escaped attention. From a consideration of the usual habitat of these creatures it appeared likely that a marked humidity reaction might exist. The experiments described below give an account of this reaction, its character and mechanism.

MATERIAL AND METHODS

The species chosen for study was *Peripatopsis moseleyi* (Wood-Mason). This species occurs in moderate numbers in many Natal forests and is invariably found in damp places, usually underneath old decayed logs of wood or burrowing in their substance. In the laboratory the animals were kept in shallow photographic dishes filled with pieces of decayed wood. These were sprayed with water once a week. The dishes were covered at one end with a glass plate and at the other with perforated zinc sheeting. The gradient of moisture thus produced lessened the danger of fungal infection, which is common in damp cultures.

The *alternative chamber* described by Gunn & Kennedy (1936) was used to establish the presence and nature of the humidity reaction. In this apparatus the animal is presented with a choice of two humidities. The preference for one or the other may be quantitatively estimated by determining the distribution of animals in the two halves of the chamber when an equilibrium has been reached. The desired humidities were maintained by solutions of potassium hydroxide of suitable concentration.

The nature of the experimental material necessitated certain modifications of the procedure employed by Gunn (1937). Only one animal could be used in each chamber, since a strong tendency to aggregate was evident when several animals were present. Secondly, the usual methods of activation could not be employed; light, as used by Kennedy (1937), was ineffective, while mechanical stirring was undesirable as the animals reacted by ejecting slime in which they frequently became entangled. It was found that activation could be brought about simply by rotating the false floor of the chamber so that the animal was brought back into the central region of the gradient. The direction of rotation was such that the animal was always turned

through more than 90°, but never more than 180°. Experiments with cobalt thiocyanate paper (Solomon, 1945) as a humidity indicator showed that the effect of this procedure on the gradient was either very small or else transient, lasting for less than 3 min.

For each experiment ten alternative chambers were used, one animal being introduced into each. One hour was allowed for the initial burst of activity to die down, after which the animals were transferred to the middle of the gradient by rotating the false floor. After a period the distribution of the animals was noted and they were moved back once more to the middle of the gradient. This process was continued until 130–250 position records were obtained. To assess the period of time taken for the complete establishment of the reaction, readings of distribution were taken initially every 3 min. This was continued until the ratio of animals in the wet to animals in the dry remained constant for five consecutive readings. This period was observed from five to eight times in each experiment and the mean value was found. Subsequently this period of time was allowed to elapse between moving the animals to the centre of the gradient and noting their positions.

It was not possible to run parallel control experiments, but such experiments, in which the humidity was the same on both sides of the chamber, were made at four different humidities. The results obtained did not differ significantly from those expected in the complete absence of any reaction.

Unfortunately Edney hygrometers were not available until almost all experiments were completed. At the end of each experiment the potassium hydroxide solutions were collected separately from the two sides of each chamber and the mean density for each side was determined. After the Edney hygrometers had been received, a series of chambers with hygrometers was set up and allowed to stand for 20 hr. At the end of this period the humidity in the two halves of each chamber was read on the hygrometers, the potash in each half was pooled and its density determined. From these observations it was possible to evaluate the humidity extremes above the false floor in any gradient. The values of the humidity gradients quoted below have been assessed in this manner. In uniform conditions the humidity has been directly determined from the density of the potash solutions.

The intensity of the reaction (I.R.) is expressed as the excess percentage of animals on the wet side, or $100\,(W-D)/(W+D)$, where W is the number of animals in the wet and D the number in the dry. An animal lying in an area 1 in. (2·54 cm.) on either side of the midline was recorded as being neither in the wet nor the dry and was not included in the calculation of I.R.

A uniform humidity chamber was employed to determine the effects of humidity on various functions such as speed and frequency of locomotion. This is identical with the alternative chamber except that a uniform humidity prevails throughout the dish. The false floor was marked out in inch squares. This allowed the position of the animal to be recorded at any moment. From such position records the animals' tracks could be drawn. The position of the posterior end of the animal was taken as representing that of the animal as a whole, for it was found that weaving movements of the anterior segments, introducing many small deviations, served only to

complicate the tracking. The resultant path is more clearly represented by the tail track. Records were made every 15 sec. and the track was subsequently drawn on squared paper.

Speed of locomotion was determined by measuring the distance between successive time marks with an opisometer. The method of determining rate of turning has been described by Ullyott (1936). Deviations imposed upon the animal by the circular nature of the apparatus have been disregarded. Since the speed was found to vary with humidity, it was undesirable to measure the rate of turning in degrees per minute. Instead it has been expressed as degrees per centimetre track. This unit will be referred to as 'angular deviation'. Locomotory activity was determined by recording at 5 min. intervals over a period of 2 hr. Animals were classified as active when showing definite locomotory activity, inactive if there was a complete absence of movement and virtually inactive in all intermediate conditions.

An *alternative air-current chamber* was employed to establish the presence or absence of a klinokinetic reaction. The apparatus consisted of a uniform humidity chamber through which a steady current of air could be passed. The current was introduced through tubes which opened at four equally spaced points beneath the false floor; the outlet was in the roof of the chamber. The rate of flow of air was about 300 ml./min. The air current could be passed through concentrated or dilute potash solutions before entering the chamber. This provided currents of low and high humidity which could be alternated at will. In preliminary experiments the changes of humidity on reversing the air current were followed by means of a dew-point hygrometer whose cup was placed just above the false floor. The results showed that the change from one humidity to another was complete in less than 5 min. To observe whether there was effective replacement of one gas by another under such conditions, an air current charged with nitric oxide was passed into the chamber. No marked inequalities of concentration of nitrogen peroxide could be seen. The gas passed as a level cloud through the false floor and displacement was complete in 3–5 min. The conditions of these experiments conform fairly closely to those under which the adaptive element of klinokinesis was first demonstrated (Ullyott, 1936), in that the stimulus is non-directional and the change of level of stimulation, although by no means instantaneous, is fairly abrupt. Any adaptational phenomenon, unless very rapid, should be detectable.

The experimental procedure employed was as follows: Air, conditioned to a fairly high relative humidity (R.H.) was blown through the chamber for 2 hr. An animal was then introduced into the chamber and tracked for 25 min. The air current was then switched so that air of low R.H. passed through the chamber. Tracking was continued for a further 25 min. and then finally the current was switched back again to the original humidity and tracking continued for a final 25 min. period. The track records were divided into 10 min. intervals and the speed and angular deviation were determined for each period. Unfortunately, experiments starting with a lower humidity could not be undertaken owing to the adverse effect on the animals of prolonged exposure to dry conditions.

While this apparatus permits a fairly rapid change in ambient humidity without

mechanical disturbance, it is open to the objection that the air current may modify the behaviour of the animal. In practice there appeared to be no significant difference between the values obtained for speed and angular deviation in the alternative current chamber and those observed in still air at the same humidities. The results in the two chambers are set out in Table 1.

Table 1. *Comparison of speed and angular deviation in still and moving air*

	Air state	Relative humidity	
		73 %	42 %
Speed (cm./min.)	Still	17·4	19·0
	Moving	15·4	20·8
Angular deviation (deg./cm.)	Still	8·2	3·5
	Moving	8·3	3·6

Note. The values for still air have been obtained by interpolation from the data presented in Figs. 3 and 6.

Nevertheless, to test this objection a further series of observations was made using a *double-humidity chamber*.* In this the false floor is supported by four peripheral and one central dish. Immediately above the latter a small watch-glass was inverted. The central dish and watch-glass were of the same diameter, so that they formed a small enclosed chamber. Attached to the watch-glass was a metal rod which projected through a small hole in the chamber roof. By means of this rod the watch-glass could be raised and suspended beneath the glass roof. Chambers were set up with the peripheral dishes containing dilute potash solution so as to give a high R.H. in the main chamber. The central dish contained a calculated excess of concentrated potash solution. The excess served to correct for any diffusion from the main chamber and thus to minimize humidity changes in the central chamber. The dishes were allowed to stand for 12 hr. before use. An animal was then confined in the central chamber. This operation necessitated removing the glass lid, but could be performed in a few seconds. The apparatus was then left for a further half an hour to condition the animal and allow the humidity conditions to be re-established. After this period the watch-glass was raised and suspended beneath the roof and the movements of the animal were recorded. The experiment could be repeated using reversed humidity conditions between the chambers. The advantage of this type of apparatus in the study of adaptation in angular deviation is that the level of stimulation is changed almost instantaneously provided the animal is active, but we found that in this simple form it was unsatisfactory owing to the thigmotactic behaviour of *Peripatopsis*. It was not possible to raise the watch-glass without disturbing the animal mechanically, an event which it was particularly desired to avoid.

A *divided alternative chamber* was used to determine the nature of the directed reaction which was found. It consisted of an alternative chamber which had been divided into two halves by glass partitions above and below the false floor. Com-

* Our thanks are due to Dr D. L. Gunn who suggested the principle of this apparatus.

munication between the two halves of the chamber was afforded by four semicircular apertures of radius 2 mm. These were cut out of the lower edge of the upper plate and were equally spaced along its edge. A medium-sized *Peripatopsis* could pass fairly easily through such a hole.

Unless otherwise stated all experiments were carried out at 25·5 ± 0·3° C. A single neon lamp was used as a source of light. Preliminary experiments showed the absence of any reaction towards such a stimulation, but as a precautionary measure the alternative chambers were arranged symmetrically around the light source and rotated through 180° half-way through each experiment.

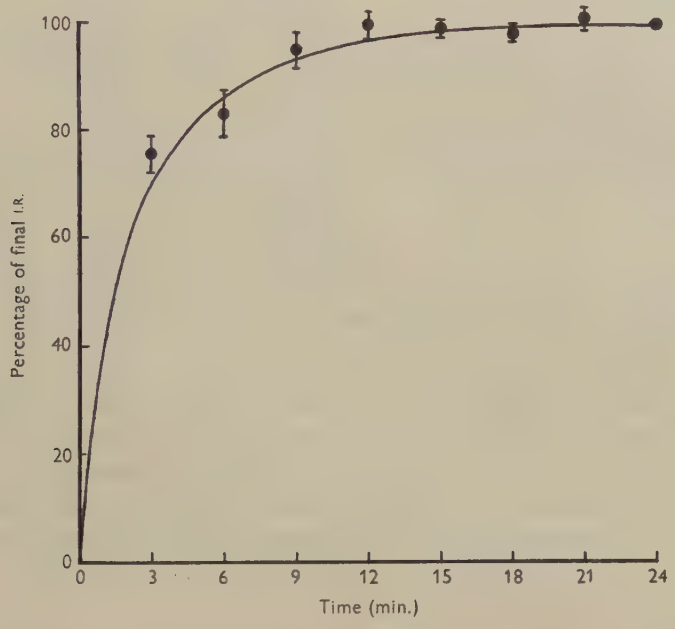

Fig. 1. The development of the humidity reaction with time. The vertical lines through the points indicate the magnitude of the standard errors.

RESULTS

The reaction to gradients of humidity

When animals were activated in the chamber a period of about 15 min. usually elapsed before an equilibrium was reached. In many gradients the time taken for the development of the reaction to its full extent was observed. These results were examined to see whether the reaction developed more rapidly in some gradients than in others. No significant differences in time of development were found. The results for all humidity gradients have therefore been summed and the resulting curve is shown in Fig. 1. This is very similar to that found by Pielou & Gunn (1940) for *Tenebrio molitor* L., but equilibrium is reached slightly earlier in *Peripatopsis*. It differs markedly from the value of 1 hr. found by Bentley (1944) for *Ptinus tectus* Boie.

A study of the I.R. was undertaken to determine the preferred or eccritic humidity and to see how the I.R. varied with the range and absolute value of the humidity gradient. The reaction of an animal to humidity may be conditioned either by relative humidity as in *Culex fatigans* (Wiedemann) (Thomson, 1938) and *Tenebrio molitor* (Pielou & Gunn, 1940) or by saturation deficiency as in *Agriotes* larvae (Lees, 1943). Dakshinamurty (1948) finds *Musca domestica* L. to react to both. These possibilities may be distinguished by determining the I.R. over the same range of R.H. at different temperatures, since under these conditions the ranges of saturation deficit will be different. The results of such experiments are shown in Table 2. It will be seen that with a decrease in temperature and consequent fall in

Table 2. *The effect of temperature on the intensity of the reaction*

Temperature (° C.)	25·5	17·0	
R.H. gradient (%)	90–61	92–63	
Saturation deficiency gradient (mm.)	2·5–9·5	1·0–5·8	
Exp.	Excess percentages		*P*
1	94·7 ± 1·8	86·4 ± 3·0	< 0·02
2	95·1 ± 1·7	85·6 ± 2·7	< 0·005
Summed	94·9 ± 1·2	86·0 ± 1·9	< 0·001

Note. *P* is the probability that the two samples were drawn from the same population.

the range of saturation deficiency, the I.R. falls. In these experiments it was further observed that the time taken for the full development of the reaction was not affected by temperature. It seems therefore unlikely that the fall in I.R. with temperature is due simply to a decrease in activity. While the possibility of some other modifying effect of temperature cannot be definitely excluded, these results suggest that the reaction is governed by saturation deficiency rather than R.H. The difficulty of maintaining low constant temperatures precluded the extension of these observations.

Table 3 shows the intensity of reaction for various humidity gradients at 25·5° C. In Fig. 2 the I.R. is plotted against the highest available R.H. From these results it will be seen that there is a slight but definite reaction away from complete saturation, although over the greater part of the range the reaction is away from the drier conditions. There is thus at this temperature an eccritic humidity of about 98% resulting from the expression of these opposed tendencies. The absolute value of the I.R. is dependent on the range of the humidity gradient as well as on the higher R.H. It will further be noted that there is probably a complete absence of reaction at low humidities. Experiments in a very dry atmosphere could not be made owing to the high mortality which occurs under such conditions.

The humidity receptors

The structure of the dermal sense organs of *Peripatopsis moseleyi* has been described by Manton & Heatley (1937). Three types have been recognized. Thin hair-like sensilla occur on the antennae. Over the general surface of the body are

further sensilla which differ from those on the antennae in having a comparatively thick cuticular covering. A third type is found on the lips and tongue. These differ from the previous types in projecting only slightly above the general surface of the skin and in being open at their tips.

Table 3. *The intensity of reaction for various humidity gradients at* 25·5° C.

R.H. range	No. of observations	Excess percentage	Standard error
99·8–99·0	356	−19·7	2·1
99·8–98·2	163	−23·9	3·4
97·0–92·0	85	3·5	2·0
97·0–88·3	137	38·7	4·2
97·0–82·5	114	38·6	4·6
95·0–78·0	76	52·6	5·7
93·5–74·5	291	89·7	1·8
92·0–75·8	165	90·3	2·3
90·5–60·5	217	99·1	0·7
90·0–58·0	92	100·0	0·0
87·0–48·0	165	100·0	0·0
82·2–72·3	113	59·3	4·6
80·5–76·8	153	16·3	3·0
75·8–57·3	174	80·5	3·0
71·5–32·2	150	93·3	2·0
68·0–59·0	132	43·9	4·3
63·0–52·5	175	32·6	3·6
59·5–44·0	91	42·9	5·2
52·0–22·3	74	59·5	5·7
41·7–20·0	44	27·3	6·7
Controls	407	0·7	0·4

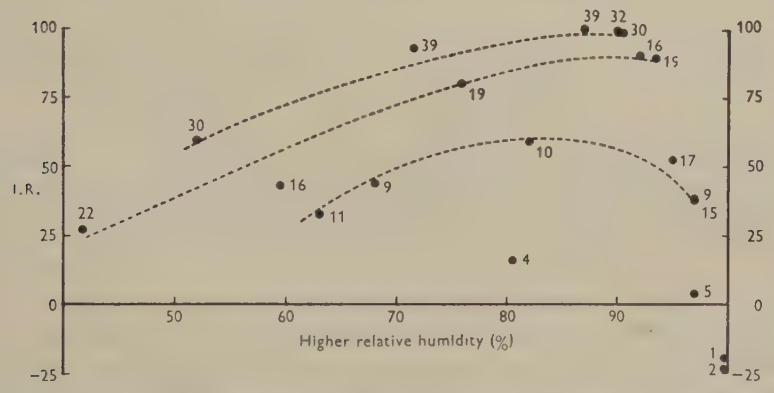

Fig. 2. The intensity of the humidity reaction plotted against the higher R.H. available. The figures by each point indicate the range of the humidity gradient. Rough isopleths corresponding to gradients of 10, 20 and 30% R.H. are shown as broken lines.

To locate the position of humidity receptors it is necessary either to amputate the organ on which they are borne and show a subsequent change of behaviour (Pielou, 1940; Lees, 1943; Wigglesworth, 1941) or to render the organ functionless in some other way (Begg & Hogben, 1946). It was found possible to remove the antennae without otherwise damaging the animals; specimens lacking their antennae survived under laboratory conditions for a period of over 6 months. For experimental

purposes a recovery period of at least 48 hr. was allowed after the operation. Various methods were tried to render the sensilla of the body functionless. The only successful procedure was that of covering the body with vaseline. The vaseline was first smeared on a glass plate. This was warmed and then pressed against the region of the animal which was to be studied. A little fluorescein was mixed with the vaseline so that the exact extent of the area covered could be determined by examination in ultra-violet light. Only a portion of the body surface could be treated at any one time, for if the whole surface was covered the animal died rapidly. The buccal sense organs were also treated with vaseline applied on a fine glass rod which was inserted into the buccal cavity. Before this operation the animals were immobilized by cooling to 0° C. for 10 min.

The antennal receptors

The effect of removing both antennae on the I.R. is shown in Table 4. It will be seen that over the range 95–78% R.H. there is no significant difference between the I.R. of normal and operated animals. It is unlikely that this negative result is due to incomplete extirpation, as examination showed that the sensilla are most concentrated towards the tip of the antennae and that there are but few near its root. With a range of 99·8–98·2% R.H. the result is different. Here the reaction away from the wet is abolished, the I.R. of the antennectomized animals not differing significantly from the value obtained in control experiments where there was no gradient.

Table 4. *Effect of removal of the antennae on the intensity of reaction*

Humidity range (% R.H.)	Controls		Experimental		*P*
	N	Excess percentage	*N*	Excess percentage	
95·0–78·0	72	52·6 ± 5·7	73	53·3 ± 5·7	> 0·9
99·8–99·2	163	−23·9 ± 3·4	181	2·8 ± 1·2	< 0·001

Note. *N* represents the number of observations. *P* is the probability that the two samples are drawn from the same population.

These results suggest that the receptors mediating the reaction away from complete saturation are not identical with those responsible for the reaction away from dry conditions. The former, but not the latter, would appear to be situated on the antennae. Confirmatory evidence for this suggestion will be presented later in a discussion of the mechanisms of the humidity reaction. The existence of an eccritic humidity of about 98% R.H. at 25·5° C. thus appears not to be due to the activity of a single type of receptor imposing a behaviour pattern by which the animal will aggregate at some humidity between saturation and dryness, but rather to be the resultant of two opposed reactions mediated by different receptor organs.

There is no evidence that the antennal humidity receptors are to be identified with the antennal spines.

The sensilla of the general body surface

All experiments were carried out in alternative chambers with a humidity gradient of about 94–75 % R.H. The controls were subjected to the same treatment as the experimental animals except that the glass plates were not vaselined. The results obtained by treating different areas are set out in Table 5. It is clear that treating the dorsal and lateral surfaces had no effect on the response. On the feet of *Peripatopsis* are heavy concentrations of sensory spines. These are mounted on pads on the median ventral surface of the appendages. Vaselining the feet and ventral surface can also be seen to be without effect on the I.R. It is apparent that attempts to identify the receptors by these methods were unsuccessful. However, experiments made with the divided alternative chamber indicated that humidity receptors are probably widely distributed over the body. These results will be discussed later.

Table 5. *Experiments on the identification of humidity receptors*

Region treated with vaseline	Controls		Experimental		*P*
	N	Excess percentage	*N*	Excess percentage	
Mid-dorsal surface	78	94·9 ± 2·4	74	89·2 ± 3·6	> 0·1
Lateral surfaces	80	60·0 ± 5·5	76	68·4 ± 5·3	> 0·2
Feet and ventral surface	370	84·3 ± 1·9	319	82·5 ± 2·1	> 0·6
Mouth and buccal cavity	79	79·8 ± 4·5	63	81·0 ± 5·0	> 0·8

Notes. All experiments were made in gradients of about 95–75 % R.H., but an identical gradient was not used for each pair of experiments. *N* is the number of observations. *P* is the probability that the samples are drawn from the same population.

A negative result was also obtained for the buccal sense organs.

The mechanism of the reaction

The behaviour patterns which may bring about an aggregation of animals in response to diffuse stimuli have been considered by Fraenkel & Gunn (1940). They consider that there may exist four types of reaction which will produce such a response. We have approached the problem with these views in mind.

Orthokinesis

The simplest mechanism proposed is described as orthokinesis. This is an undirected reaction in which speed or frequency of locomotion is dependent on the intensity of stimulation. To determine whether this mechanism was present the speed and activity of animals were studied at various humidities in the uniform chamber. The effect of humidity on speed is shown in Fig. 3. It will be seen that speed is minimal at 98 % R.H., rising sharply on either side of this point, but levelling off at lower relative humidities. Humidity influences the activity of the animals as well as their speed of locomotion. Fig. 4 shows the effect. Activity and inactivity bear much the same relation to humidity as does speed, but virtual inactivity is independent of humidity within the limits of variability.

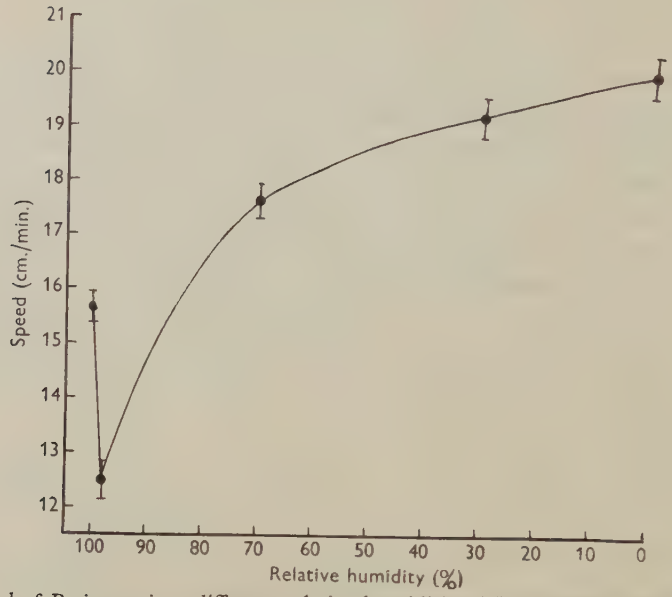

Fig. 3. Speed of *Peripatopsis* at different relative humidities. The vertical lines through the points indicate the magnitude of the standard errors.

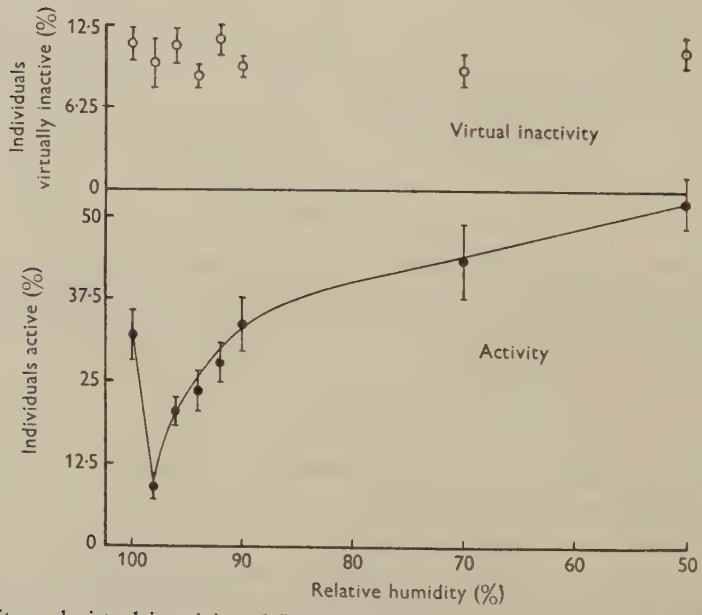

Fig. 4. Activity and virtual inactivity of *Peripatopsis* at different relative humidities. The vertical lines through the points indicate the magnitude of the standard errors.

From Figs. 3 and 4 it is clear that the high level of speed and activity found at very high humidities will be partly or wholly responsible for the observed reaction away from saturation. It has previously been shown that removal of the antennae

abolishes the reaction away from saturation in a gradient of 99·8–98·2% R.H. It is therefore to be expected that animals without antennae would neither move faster nor show greater activity at saturation than at 98% R.H. This expectation has been confirmed. Fig. 5 and Table 6 compare the behaviour of normal and antennectomized

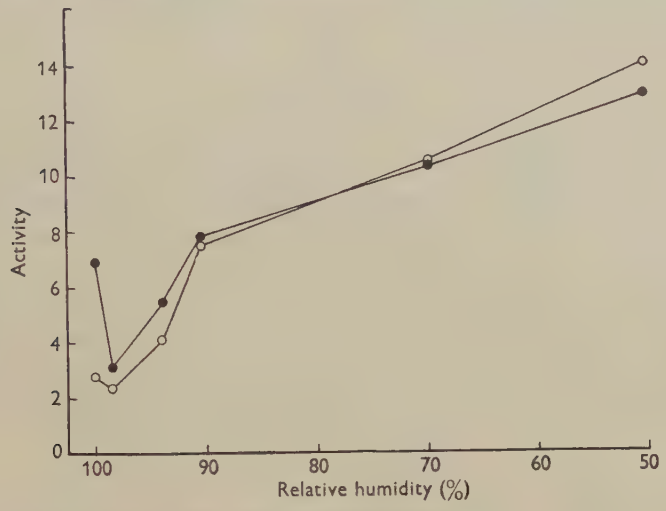

Fig. 5. Activity, at different humidities, of normal *Peripatopsis* (black circles) and of animals lacking antennae (open circles). The unit of the ordinate is given in the note to Table 6.

Table 6. *The effect of removal of the antennae on activity and speed*

Activity

Relative humidity (%)	Control	Experimental	*P*
100·0	6·93 ± 0·91	2·77 ± 0·75	< 0·001
98·5	3·08 ± 0·59	2·38 ± 0·70	> 0·4
94·0	5·54 ± 1·07	4·16 ± 0·84	> 0·3
90·5	7·84 ± 1·61	7·54 ± 1·52	> 0·8
70·0	10·53 ± 1·81	10·69 ± 1·06	> 0·9
50·5	13·22 ± 1·55	14·31 ± 0·98	> 0·5

Note. In each case thirteen pairs of animals were observed on twenty-four occasions at intervals of 5 min. The values given are means of the individual results. *P* is the probability that the samples were drawn from the same population.

Speed

Relative humidity (%)	Control	Experimental	*N*	*P*
100·0	15·66 ± 0·28	11·84 ± 0·36	26	< 0·001
70·0	17·62 ± 0·30	18·30 ± 0·65	20	> 0·3

Note. The control values are those given in Fig. 3. The speed of the experimental animals was measured over track lengths of 5 min. duration. *N* is the number of track lengths averaged.

animals at different humidities. It will be seen that the activity of antennectomized animals at saturation is no greater than at 98% R.H., although the general level of activity for both normal and experimental animals is the same at lower humidities. A similar effect for speed of locomotion is shown in the table.

It thus appears that *Peripatopsis* displays a marked orthokinesis in both speed and activity. In mediating the reaction away from the dry this response is most effective between 85 and 98% R.H. At the same time the experiments with antennectomized animals have shown that orthokinesis is also partly responsible for the reaction away from saturation, and thus it clearly plays an important part in giving an eccritic humidity of about 98% at 25·5° C.

Klinokinesis

A second method of aggregation in a non-directional gradient depends upon an increase in angular deviation with increased intensity of stimulation. Adaptation to the increased stimulation is an essential adjunct. This type of response has been demonstrated most clearly in the reaction of *Dendrocoelum lacteum* Oerst. to light (Ullyott, 1936).

In the study of this type of reaction the angular deviation was first determined at various humidities in the uniform chamber. The results are shown in Fig. 6. It will be seen that there is a significant decrease in the angular deviation with decreasing humidity. This result is different from that of Ullyott, where there was the same basal angular deviation at all intensities of illumination.

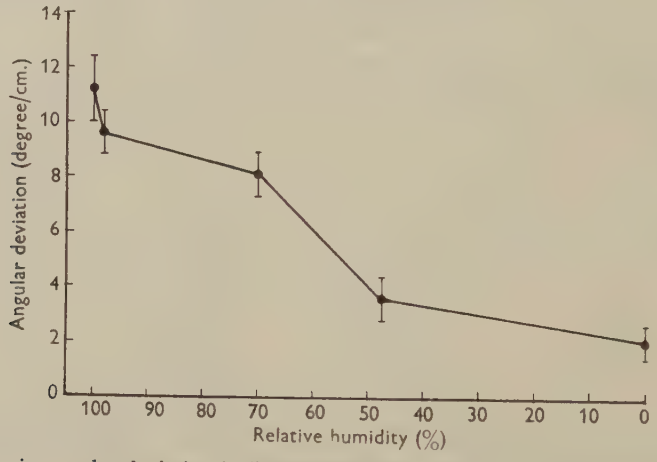

Fig. 6. Variation in angular deviation in *Peripatopsis* with humidity. The vertical lines through the points indicate the magnitude of the standard errors.

There was, however, some evidence of an adaptation in *Peripatopsis*, although the decrease in angular deviation with time was very irregular. It was thought that this might be an expression of some activation following the introduction of the animals into the chamber. To test the validity of this suggestion, experiments were carried out in the alternative air-current chamber so as to eliminate the effect of sudden mechanical disturbance. The results obtained are presented in Table 7 and Fig. 7. The experiments show that the angular deviation is maintained at a high level for 25 min. after the humidity of the air current is changed from 42 to 73% R.H. There is no evidence of adaptation and, moreover, the basic angular deviation varies with stimulation intensity, an effect not found in true klinokinesis.

Table 7. *The effect of humidity on speed and angular deviation in the alternative current chamber*

Time	Relative humidity	Speed	Angular deviation	*N*
1st 10 min.	73	14·9 ± 1·4	6·7 ± 1·2	17
2nd 10 min.	73	15·2 ± 1·9	5·8 ± 2·1	15
3rd 10 min.	73/42	15·3 ± 1·4	4·9 ± 1·1	17
4th 10 min.	42	20·9 ± 1·8	3·4 ± 0·8	21
5th 10 min.	42	21·8 ± 1·3	3·7 ± 0·5	21
6th 10 min.	73	16·4 ± 1·5	7·6 ± 1·8	15
Last 15 min.	73	16·8 ± 1·0	8·8 ± 1·0	19

Note. *N* is the number of animals used to determine each value. The value of *N* varies as some animals were completely inactive during a period of observation.

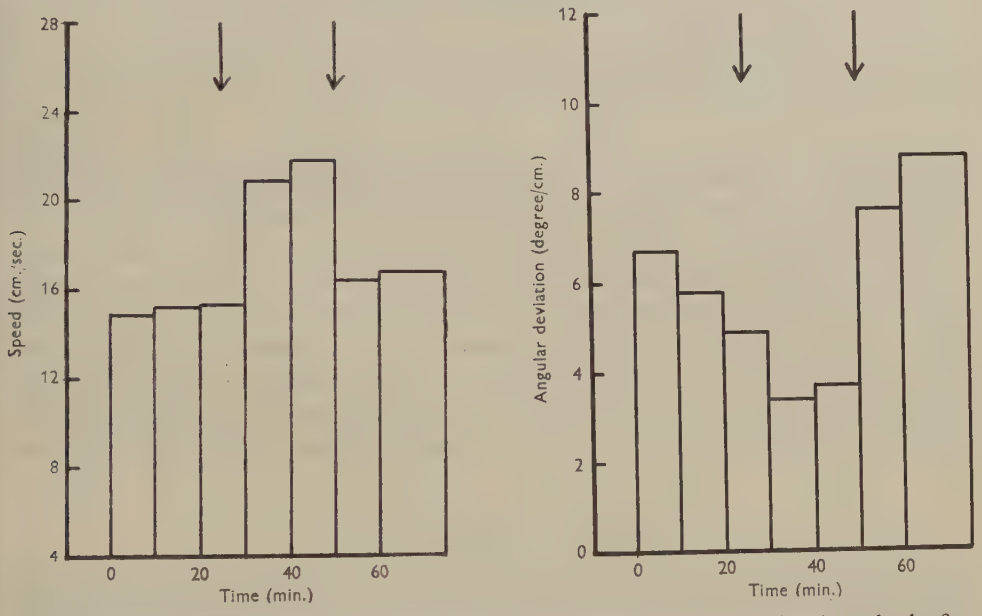

Fig. 7. Variation in speed and angular deviation in the alternative air-current chamber. At the first arrow the ambient humidity is changed from 73 to 42 % R.H. and at the second from 42 to 73 %. Compare with Table 7.

As has been stated above, these experiments are open to the objection that the pattern of behaviour may be modified by the air current. It is also possible that the rate of change of stimulation was so slow that adaptation might have proceeded as rapidly as the humidity changed. To check these results, experiments were carried out in the double-humidity chamber. The results obtained are set out in Table 8. Two control experiments were made in which both central and main chambers were adjusted to the same humidity. It will be seen that, while the angular deviation is reasonably constant at 80 % R.H., in the dry chamber the value falls gradually to a level not significantly different from that observed in the uniform humidity chamber (Fig. 6). This suggests that some element of mechanical disturbance is introduced in

releasing the animals from the central chamber. The experimental results show the same pattern as the controls. This might be due to one or more of a number of causes; the important observation, however, is that where the animal moves from the dry to the wet the high level of angular deviation appears to be immediately established and to be maintained. The whole pattern is thus distinct from klinokinesis with adaptation.

Table 8. *The effect of humidity on angular deviation in the double-humidity chamber*

Time	Relative humidity				
	Central chamber	100	15	80	0
	Main chamber	0	80	80	0
1st 10 min.		8·8	8·5	8·6	5·5
2nd 10 min.		3·4	9·8	—	4·0
3rd 10 min.		5·1	7·8	9·8	2·4
No. of experiments		6	12	9	5

To make a rough assessment of the possible importance of these effects in contributing to an aggregation in the wet, a hypothetical track was drawn of an animal in an alternative chamber of which one-half was at 75% R.H. and the other at 40%. Appropriate values of mean angular deviation were used in each half of the chamber. The direction of turn was randomized by spinning a coin. The I.R. was calculated from the track lengths in the wet and in the dry and was found to be 5%. It is thus clear that the turning movement, while slightly enhancing the wet preference, is not an important factor under these conditions. The effect will be increased in a more extreme gradient, but is unlikely to make a major contribution to the total reaction.

The directed reaction

A formula devised by Thorpe, Crombie, Hill & Darrah (1947) makes it possible to assess approximately the intensity of a reaction mediated by orthokinesis when the relation between mean speed and humidity is known. When this formula is applied to the data obtained from *Peripatopsis* it appears that the reaction away from saturation can be completely accounted for by orthokinesis. Thus over the humidity range of 99·8–99·0% the expected value of the I.R. is 35·2%, which is more than adequate to account for the observed value of 19·7%. Over other parts of the gradient orthokinesis makes only a partial contribution to the reaction as a whole. Thus over a gradient of 39% R.H. with a higher relative humidity of 87%, the expected I.R. is 38·9%, while the observed value is 100%. Since the klinokinetic mechanism is clearly insufficient to account for this difference, a search was made for a directed reaction.

Two types of directed reaction are considered by Fraenkel & Gunn (1940) to be possible in a gradient. In the one, klinotaxis, successive intensity comparisons are made by regularly alternating deviations of a part or the whole of the body. The other,

tropotaxis, is dependent on the simultaneous comparison of the intensity of stimulation of two separate receptors or groups of receptors on either side of the body. For the analysis of the directed reaction the divided alternative chamber was used. An animal was introduced into one-half of the chamber and its behaviour noted. It moved in a random fashion and would eventually reach one of the apertures leading to the other half. Here one of three things invariably occurred:

(i) The animal went straight through the aperture and into the other half without hesitation.

(ii) The animal moved partly through the aperture, locomotion ceased abruptly, and after a certain interval of time the animal backed into the half whence it came.

(iii) As in (ii), the animal ceased to move for a time, but then, instead of backing out, it continued on its way into the other half of the chamber.

The behaviour described in (ii) is an 'avoiding reaction', while that shown in (i) and (iii) was considered as 'no reaction'. Several different animals were used in a single experiment and each was observed for a period of 1–2 hr. The results are expressed as the percentage of the total number of trials which showed an avoiding reaction.

The first experiment was done with R.H. of 0 and 100% in the two halves of the chamber. The results are given in Table 9. It will be seen that the percentage

Table 9. *Experiments on the directed reaction in the divided chamber*

Relative humidity gradient	Treatment	Avoidance of dry		Avoidance of wet	
		%	*N*	%	*N*
100–0	None	67·7 ± 8·4	31	6·7 ± 6·7	15
50–50	None	20·0 ± 10·4	12	20·0 ± 12·7	10
80–50	None	66·7 ± 15·7	9	14·3 ± 9·4	14
80–50	Posterior regions smeared	50·0 ± 15·8	10	66·8 ± 11·1	18

Note. *N* is the number of trials.

avoidance for animals going from the wet to the dry is ten times that of animals moving in the opposite direction. In only one instance was an avoiding reaction to the wet observed, and this was made by an animal which had just been introduced into the chamber. The experiments were repeated using humidities of 80 and 50% on the wet and dry sides respectively. In this case the percentage avoidance of the dry was 67 and of the wet 14. As a control the experiment was repeated with 50% R.H. on both sides of the barrier. The percentage avoidance was the same in both directions.

These results show that the directed reaction cannot be klinotactic in the strict sense of the word. Once the body of the animal is passing through the aperture the anterior regions of the body will be subjected to uniform stimulation in spite of any lateral deflexions which may be made, communication between the two halves of the chamber being blocked by the body of the animal. Lateral deflexion will not therefore afford an opportunity for the comparison of different intensities of stimulation.

The experiment does not exclude the possibility that the animal may achieve orientation by comparison of intensities which are successive in time, but independent of any lateral movement. Such a klinotaxis has been shown by Lees (1943) in the larvae of *Agriotes*. The only other manner in which orientation could be carried out would be by a comparison of the intensities stimulating the anterior and posterior parts of the body. In other words by a tropotaxis based on a comparison of stimulation of anterior and posterior receptors rather than of bilaterally symmetrical ones.

To distinguish between these alternatives, experiments were carried out in which the posterior end of the animal was smeared with vaseline. This treatment was assumed to prevent evaporation and thus be to the animal equivalent to stimulation by a saturated atmosphere. The treated animals were introduced into the drier half of a divided alternative chamber in which the R.H. was 50%; the R.H. on the other side was 80%. If the directed reaction depended upon successive comparisons in time it would be expected that under these conditions the animals would preferentially pass into the moister half of the chamber. If, however, the reaction depended upon a comparison of anterior and posterior ends, it would be expected that the animal would back into the drier half. In the experiment it was found that, while the percentage avoidance of the dry by animals which had passed into the wet half of the chamber was in good agreement with the controls ($\chi^2 = 0{\cdot}54$; $P = 0{\cdot}5$), the percentage avoidance of the wet by animals moving out of the dry was 67 compared with a value of 14 for the controls ($\chi^2 = 8{\cdot}78$; $P = 0{\cdot}01$). In other words, two out of every three treated animals backed into the dry while only three out of fourteen normal animals did so. An avoiding reaction was therefore performed in spite of the fact that it had the effect of bringing the animal back into the drier half of the chamber. These results are most easily explained on the assumption that the directed reaction of *Peripatopsis* is based on a comparison of humidities at the anterior and posterior ends of the body.

This hypothesis is supported by the observation that animals whose posterior ends have been smeared with vaseline turn abruptly through angles of 180° more frequently than do untreated controls. The average number of such turns made by treated animals was $5{\cdot}5 \pm 0{\cdot}6$ in 15 min., while the corresponding value for untreated controls was $2{\cdot}5 \pm 0{\cdot}3$. Both sets of observations were made at 80% R.H. This phenomenon can probably be regarded as a novel expression of the traditional 'circus movements'.

It should be remarked that the smearing of the posterior regions of the animals caused their death within 12 hr., and that within 3 hr. of smearing the treated half of the body was rendered functionless as regards locomotion. For this reason no attempt was made to confirm these observations using an alternative chamber. In using the divided chamber the animals were allowed half an hour to recover from treatment and then observed for a period of less than 2 hr. It therefore seems unlikely that these considerations need invalidate the results.

It should also be emphasized that these experiments do not exclude the possibility that orthodox klinotaxis exists. They show that it cannot be the basis of the avoiding reaction, but the possibility remains that lateral deviations of the anterior end of the

organism assist in orientation. We have been unable to devise experiments which would enable us to test this possibility.

It may be concluded that the pattern of humidity behaviour of *Peripatopsis* includes a directed reaction which is dependent upon a simultaneous comparison of the stimulation intensity at the two ends of the body. In addition, a complex orthokinetic reaction is responsible both for a reinforcement of the directed reaction away from the dry and for an avoidance of complete saturation. There thus results a preferred humidity of about 98% R.H. at 25·5° C.

The effect of desiccation upon the reaction

A few experiments have been made to study the effect of desiccation upon the intensity of the reaction. These are summarized in Table 10. It will be seen that there is a significant reduction of the I.R. with desiccation.

Table 10. *The effect of desiccation on the intensity of the reaction*

Duration of desiccation	Controls		Experimental		*P*
	N	Excess percentage	*N*	Excess percentage	
2 hours	102	88·2 ± 3·2	92	78·3 ± 4·3	< 0·1
3 hours	104	92·5 ± 2·6	110	80·0 ± 3·8	< 0·01
4 hours	83	88·0 ± 3·6	105	54·3 ± 4·9	< 0·001

Notes. *N* is the number of observations. *P* is the probability that the samples were drawn from the same population. All experiments were made in a gradient of 93·5–74·5 % R.H.

This effect might be due to two possible causes. One is that desiccation is accompanied by a relative increase in the speed and level of activity at the higher humidities compared with the lower. This would result in a weakening of the orthokinetic contribution to the reaction and the I.R. would thus fall. The other possibility arises from the character of the humidity receptors. Evidence has been brought forward to show that these depend upon the rate of evaporation of water. It has been shown by Manton & Heatley (1937) that the rate of evaporation of water falls off with desiccation in *Peripatopsis*. It is therefore possible that the sensitivity of the humidity receptors falls and the tropotactic reaction is weakened. The orthokinetic reaction may also be less intense as a result of lowered speed and activity at lower humidities. Experiments to distinguish between these possibilities have yet to be undertaken.

DISCUSSION

The first point of interest in the behaviour of *Peripatopsis* is clearly the marked preference for a humidity of about 98% R.H. at 25·5° C. In the case of most arthropods which have been studied, a more or less clearly marked preference, either for saturation or for dryness, has been discovered. Sometimes this preference is expressed over the whole humidity range. Thus Pielou & Gunn (1940) have shown a weak but definite preference for dryness in *Tenebrio molitor* in a gradient of 5–10% R.H., while Lees (1943) has shown a preference for saturation in *Agriotes*

larvae over a range of 99·5–100% R.H. Frequently the preference is not strongly marked outside a certain range of humidity. Thus Wigglesworth (1941) has shown that *Pediculus humanis corporis* de Geer avoids humidities of 95% and above, but is indifferent to lower values. Similarly, Gunn (1937) has found *Porcellio scaber* (Latreille) to prefer humid conditions but to be indifferent in gradients within the range of 80–100% R.H.

A preferred humidity of 60–80% R.H. has been shown to exist for blood-fed females of *Culex fatigans* by Thomson (1938), while Begg & Hogben (1946) claim that *Drosophila melanogaster* Meigen has a preferred humidity of 90%; their published figures do not, however, fully support this idea. We have shown that in *Peripatopsis* the preference is the result of the opposing action of two sets of receptors. Begg & Hogben have also demonstrated the presence of two sets of receptors by their experiments on mutant *Drosophila*. They have shown the presence of antennal receptors which mediate a reaction towards the wet and of other unidentified receptors mediating a reaction towards the dry. They point out that there is the possibility that the two opposed behaviour patterns may be dependent upon the stimulation of identical types of receptors which have different nervous connexions. This possibility cannot be excluded in *Peripatopsis*.

The existence of a clearly marked eccritic humidity recalls the existence of temperature preferences in many animals. It was felt that it would be of interest to see how far the two cases are parallel. Unfortunately, the methods by which preferred temperatures are attained have been little studied. Analysing earlier work Fraenkel & Gunn (1940) consider that klinokinetic reactions away from both extremes play an important part. Wigglesworth (1941) has found this to be so in *Pediculus*, but in two other recently investigated cases (*Agriotes*, Falconer, 1945; *Ptinus*, Gunn & Walshe, 1942) this possibility seems to have been excluded, and aggregation is attributed in both instances to a 'shock reaction' which has not been further analysed. These results find a parallel in *Culex*, where Thomson (1938) has described a 'hygrophobotactic' response away from both extremes of humidity. The condition in *Peripatopsis*, where the avoidance of one extreme is dependent on orthokinesis alone, has no parallel in investigated examples of temperature preferences.

The biological significance of this eccritic humidity for *Peripatopsis* may lie in the susceptibility of this animal to fungal infection. It is likely that by avoiding conditions of complete saturation, the animal is assisted in avoiding this hazard.

A second feature of interest is the decline in the I.R. with desiccation. Where this effect has previously been examined in animals showing a dry preference, that preference has been weakened as in *Locusta migratoria migratorioides* R. & F. (Kennedy, 1937) and *Culex* (Thomson, 1938), or the reaction has been reversed as in *Blatta orientalis* L. (Gunn & Cosway, 1938), *Ptinus* (Bentley, 1944) and *Ixodes ricinus* L. (Lees, 1948). Cases are also known where desiccation initiates a reaction towards the wet where previously none could be detected. In only one case have details of the effect of desiccation on an animal showing a wet preference been published. This is in *Drosophila* (Begg & Hogben, 1946), where the wet preference is strengthened. It will be seen that all these cases differ from that of *Peripatopsis* in so far as the behaviour following desiccation assists these other animals to a greater

or less degree to reduce water loss. In its unteleological behaviour *Peripatopsis* is at the moment unique. It will require further investigation to see whether this effect is indeed a concomitant of the evaporimeter type of receptor, as has been suggested above, and whether compensating nervous mechanisms have been developed in other forms.

SUMMARY

1. The behaviour of *Peripatopsis moseleyi* (Wood-Mason) towards humidity has been investigated. At 25·5° C. the animal is found to have a preferred relative humidity of about 98%.

2. Humidity receptors mediating a reaction towards the wet occur over the general surface of the body. Receptors mediating a reaction towards the dry occur on the antennae. The receptors on the body surface probably depend for their functioning upon the rate of evaporation of water.

3. The reaction towards the wet is effected by an orthokinesis involving both speed and activity. This is enhanced by a tendency to turn more frequently in the wet than in the dry. There is further a directed reaction which depends upon the simultaneous comparison of the stimulation intensities at the anterior and posterior ends of the animal.

4. The reaction away from saturation is due to a speed and activity orthokinesis.

5. The existence of a reaction away from saturation together with the opposing reactions away from dryness result in a preferred humidity just below saturation.

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THE KINETICS OF LOCOMOTION OF THE GRASS-SNAKE

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(With Sixteen Text-figures)

The propulsion of an undulating grass-snake, *Natrix* (*Tropidonotus*) *natrix*, depends on the ability of the animal to adopt a sinusoidal form and brace the lateral surface of its body against rigid external objects (Gray, 1946). Under such conditions the forces acting normally to the sides of the body provide a propulsive force equal but opposite to the frictional forces tending to restrain the motion of the animal. The present paper represents an attempt to give quantitative expression to these conclusions by observation of the forces acting against the body of a living snake.

THE FRICTIONAL FORCES OPPOSING SERPENTINE OR UNDULATORY MOVEMENT

The frictional forces operating against the body of a moving snake can be divided into two groups: (i) *ventral friction* acting between the ventral surface of the body and the ground, (ii) *lateral friction* acting against the sides of the body wherever pressure is applied by the snake to an external lateral resistance. During a typical glide, each segment of the body follows in the path of the segment next anterior to itself, and consequently both frictional components act along or parallel to the path of motion.*

An estimate of the longitudinal ventral friction acting along the path of motion can be derived from observation of the forces necessary to tow the body of a dead snake rectilinearly over a variety of plane level surfaces; data of this type are recorded in Table 1. So long as the body is moving over reasonably smooth dry wood, metal or glass, the coefficient (μ) of ventral friction (friction/weight) is of the order of 0·2–0·4. The results obtained with sandpaper of varying roughness are surprising; they show that the friction decreases with increasing roughness of ground. It is also interesting that the friction on a metal or glass surface is markedly *increased* by the presence of a film of oil.

The coefficient of lateral friction has been assessed by allowing a living snake to glide actively through a relatively close-fitting channel containing two rectangular bends (Fig. 1), one of the sections (15 cm. long) of the channel being mounted on a balance (Barclay, 1946) capable of registering the total forces acting normally and

* By appropriate muscular activity, the snake can also brace the ventral surface of the body against *transverse* friction exerted by the ground; this force is probably small in comparison with the lateral friction exerted by rigid external resistances or the longitudinal ventral friction, and will only be considered to a very limited extent in this paper (see Fig. 10 (iv) Folder III).

Table 1

Substratum	Length of snake (cm.)	Weight (W) of snake (g.)	Towing force in g.		T_A/W
			Head first (T_A)	Tail first (T_P)	
Glass (dry)	70	70	28		0·40
Glass (dry)	64	80	34		0·42
Glass (oil film)	70	70	90		1·30
Metal (dry)	70	70	24		0·34
Metal (dry)	78	180	60	60	0·33
Metal (oil film)	70	70	>60		>0·86
Wood (dry; smooth)	64	80	27		0·34
Cardboard (smooth)	67	83	20		0·24
Sandpaper (fine)	64	80	52	60	0·65
Sandpaper (medium)	64	80	52	70	0·65
Sandpaper (medium rough)	64	80	40	85	0·50
Sandpaper (rough)	64	80	35	105	0·44
Sandpaper (rough)	70	70	20		0·30
Sandpaper (rough)	66	57	25	120	0·44
Fibre mat	66	57	35	75	0·61

tangentially to the direction of the snake's motion. The ventral friction, due to the weight of the animal, was measured by towing a dead snake of comparable size rectilinearly over the bridge. The results obtained are shown in Table 2. The consistency of the values obtained for the coefficient of lateral friction is probably fortuitous, but it seems reasonable to conclude that the coefficients of lateral and

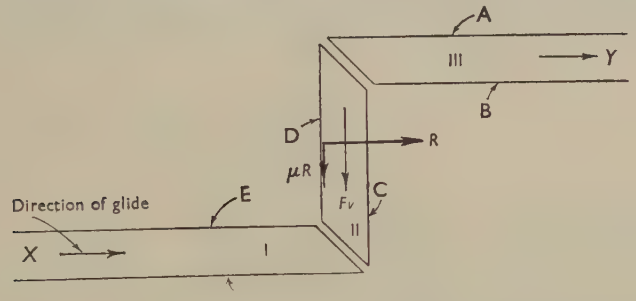

Fig. 1. Channel with two rectangular bends; the centre section (II) is mounted on a Barclay bridge registering simultaneously (i) the reaction (R) from the posterior wall D and (ii) the total friction ($\mu R + Fv$) acting on section II of the channel. The section of channel on the bridge was 15 cm. long.

Table 2

(Floor and walls of channel: dry metal)

Reaction (R) normal to surface (g.)	Total friction ($Fv + \mu R$) (g.)	Ventral friction (Fv) (g.)	Lateral friction (μR) (g.)	Coefficient of lateral friction (μ)
45	26	10	16	0·35
25	20	10	10	0·40
20	18	10	8	0·40
25	20	10	10	0·40
18	17	10	7	0·39

ventral friction are not greatly different from each other and are of the order of 0·2–0·4 for reasonably smooth surfaces.

MOVEMENT OF A SNAKE THROUGH A CLOSE-FITTING RECTANGULAR CHANNEL

In order to estimate the magnitude and distribution of the forces acting normally to the surface of the body, a dry metal channel (Fig. 2) containing two rectangular bends was employed, both walls of each of the three sections (I, II and III) being,

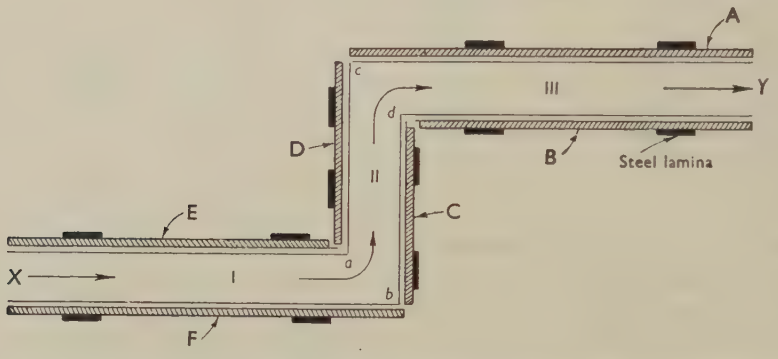

Fig. 2. Channel with two bends, the walls being mounted on vertical steel strips registering forces acting normally to the walls of the channel. When the snake was gliding towards *Y*, pressures were exerted against the walls B, D and F; no pressures were exerted against walls A, C and E. When the snake was gliding towards *X* the distribution of pressures was reversed.

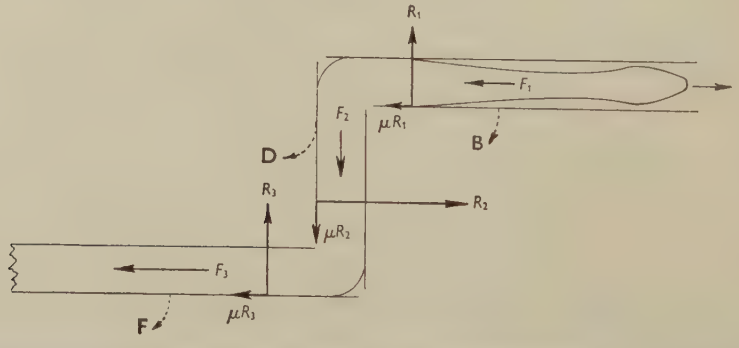

Fig. 3. Diagram showing the general distribution of all external forces acting on a snake when gliding through a channel with two rectangular bends. F_1, F_2 and F_3 represent the ventral sliding friction acting on each of the three sections of the channel. R_1, R_2 and R_3 represent the pressures exerted by walls B, D and F normal to their surfaces. μR_1, μR_2 and μR_3 represent the lateral friction associated with R_1, R_2 and R_3.

in turn, mounted independently on vertical strips of steel; the lateral displacement of these strips gave a measure of the forces acting normally to the walls of the section. If the animal was admitted at *X*, it progressed by 'concertina' movements (Gray, 1946) until the head had passed the second bend at *cd* and the right anterior surface of the body was applied to the wall B; from this point onwards the snake began to glide. Records obtained in this way showed conclusively that the lateral pressures

from the body of a snake gliding from X towards Y were invariably exerted against the 'posterior' walls B, D and F, no pressures being exerted against the 'anterior' walls A, C and E. When the snake glided from Y towards X, the lateral pressures were invariably transferred to walls A, C and E. In every case, the total length of snake enclosed by sections I and III was greater than that in section II and the pressure exerted against the wall of section II was considerably greater than that against the wall of section I or section III. Reference to Fig. 3 shows that the normal reaction (R_2) exerted on the snake by the wall D of section II provides a measure of all the frictional forces acting against the regions of the body enclosed by sections I and III, the total frictional force of these two sections being composed of the ventral components F_1 and F_3, together with the lateral friction associated with

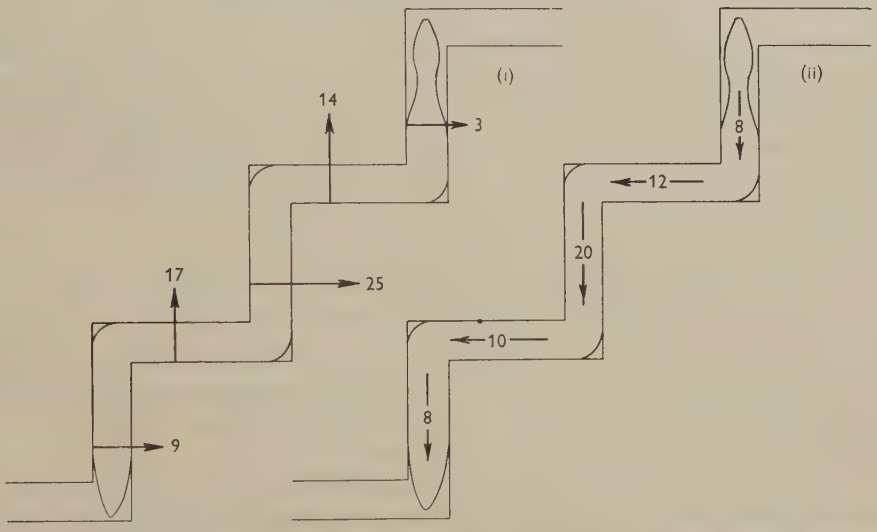

Fig. 4. Diagram showing the forces actually elicited from the walls of a zigzag channel. (i) shows the forces acting normally to the surface of the channel; (ii) shows the total friction acting against the body. The sum of the normal forces should equal that of the frictional forces; the figures actually recorded were normal forces 68 g. and frictional forces 58 g.

the forces (R_1 and R_3) acting normally to the walls. Similarly, the sum of the forces R_1 and R_3 measures the total friction acting against section II of the channel. Finally, the total ventral friction ($F_1+F_2+F_3$) is related to the weight of the animal (W) by the coefficient of ventral friction.

During a steady glide three equations must be satisfied:

$$R_2=F_1+F_3+\mu(R_1+R_3), \quad \text{(i)}$$

$$R_1+R_3=F_2+\mu R_2, \quad \text{(ii)}$$

$$F_1+F_2+F_3=\mu W. \quad \text{(iii)}$$

The total force ($R_1+R_2+R_3$) acting normally to the walls of the channel should therefore equal $\mu W/(1-\mu)$; if $\mu=0{\cdot}4$, the total force acting normally to the surface of the walls should equal two-thirds of the weight of the snake. For a snake weighing

78 g., the forces acting normally to the walls of sections I, II and III were found to be 10, 35 and 10 g. respectively, making a total of 55 g. compared with the theoretical estimate of 52 g.

Similar results were obtained when a snake was allowed to glide through a zigzag channel of the type shown in Fig. 4, one section being mounted on a bridge registering, simultaneously, pressures normal to its walls and the total friction acting along its length. The lateral and antero-posterior displacements of the bridge were amplified by mechanical levers whose movements, together with the position of the snake in the channel, were recorded photographically from the moment the snake's head entered the recording section until the tip of the tail had passed through. Fig. 4 is constructed from data of this type. For a snake weighing 80 g., the total force acting normally to its surface was found to be 68 g. On the basis of a coefficient of friction of 0·4, the ventral friction should have been 32 g. and the lateral friction 27 g., making a total of 59 g.; the total observed friction was 58 g. Undue emphasis must not be laid on the close agreement between the observed and calculated values of total friction; complete agreement between theory and observation involves an equality between the total forces acting normally to the body and the total friction; in fact, the observed forces acting normally to the body were 68 g., whereas the observed friction was 58.

THE FORCES EXERTED BY A SNAKE WHEN GLIDING PAST A SERIES OF VERTICAL PEGS

Under natural conditions, a grass-snake glides forward by bracing the sides of its body against grass, stones or other projections from the surface of the ground, the reactions from these projections yielding a resultant force equal but opposite to the ventral friction operating between the body and the ground.

The magnitudes and directions of the reactions from external objects can be determined by allowing the animal to exert its effort against a series of relatively heavy cylindrical pendulums whose displacements can be measured photographically as the animal glides along. The force exerted by the snake against each pendulum (Fig. 5) has two components—one acting normally to the surface of the cylinder and the other (lateral friction) acting tangentially to its surface.

As observed by Wiedemann (1932), a snake propels itself past a single cylindrical peg by throwing its body into an S-shaped bend and applying pressure to the anterior surface of the peg. Fig. 6 (i)* of the present paper shows this typical form, together with the magnitude and direction of the force exerted by a single pendulum against the body of the animal. For a snake weighing 85 g. and gliding over a smooth glass surface the total reaction (S) from the pendulum was 19 g. and was directed approximately along the axis of forward motion of the snake. As shown in the figure, the reaction (R) which acts normally to the surface of the body has two components; one (D) equal and opposite to the friction (Fl) acting at the surface of the pendulum, and the other (S) equal but opposite to the ventral friction (Fv). In Fig. 6 (ii) the same snake is seen gliding past two pendulums, the total reaction from one peg

* For Figs. 6, 7 and 8 see Folder I.

being 5 g. and from the other 12 g., yielding a resultant (*St*) of 16·5 g. along the axis of motion. When operating against three pendulums (Fig. 6 (iii)), the resultant reaction from the pegs yielded a forward thrust along the axis of motion of approximately 15·5 g.; when operating against a larger number of pendulums (Fig. 7) the resultant forward thrust varied from 17 to 22 g. These values represent the component of friction acting along the axis of motion and indicate a coefficient of ventral friction not less than 0·2.

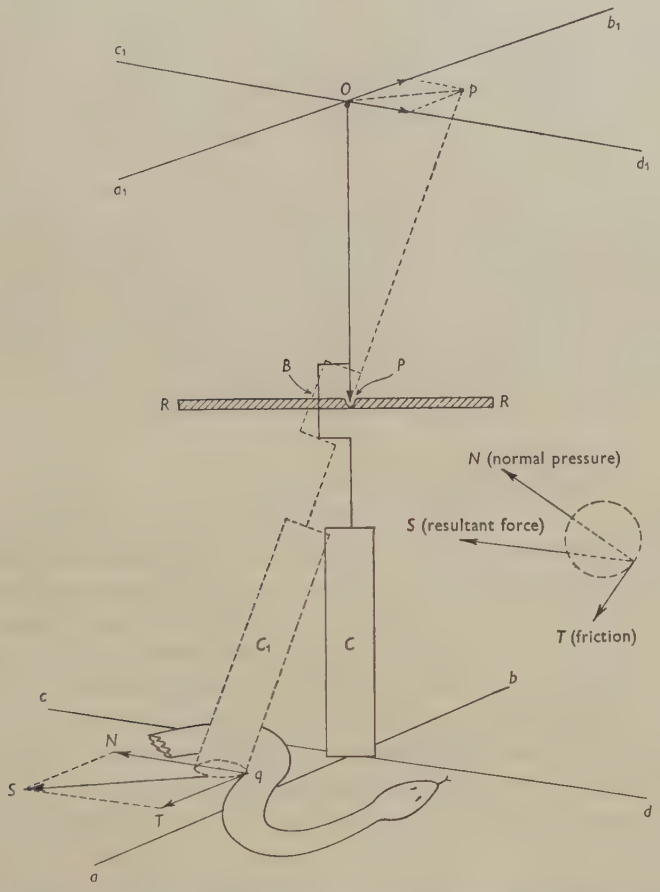

Fig. 5. Apparatus for determination of the force exerted by a snake when gliding past the surface of a vertical cylindrical peg. A relatively heavy cylinder (*C*) is suspended by a pin-pivot (*P*) to a rigid horizontal bar (*RR*); the cylinder can rotate about the pivot with respect to both its horizontal transverse axes. Rotation about its vertical longitudinal axis is restricted to 180° after which the arm *B* engages with the transverse bar. Above the pivot is a long rigid pointer *PO*, whose upper end lies at the intersection of two horizontal rectangular axes (a_1b_1, c_1d_1). When undisturbed, the centre of the base of the cylinder lies at the intersection of axes (*ab*, *cd*) which are parallel to a_1b_1 and c_1d_1. When the snake engages with the base of the cylinder, the latter is displaced with reference to the axes *ab* and *cd*; simultaneously, the pointer is displaced with reference to a_1b_1 and c_1d_1; the line *Op* is parallel and proportional to the resultant force (*S*) exerted by the snake on the cylinder.

Fig. 7 shows that as the number of external resistances is increased the total force which the snake exerts transversely to its axis of motion also increases, whereas the

resultant forward thrust remains relatively constant. The records also show that the pressure exerted against any particular resistance may vary greatly as the snake moves along, although the pressures exerted by the anterior end of the animal are usually relatively small.

If a peg were perfectly smooth, the reaction from its surface would pass through the centre of curvature of the peg and consequently the line of action of the ventral longitudinal friction must also pass through this point. Friction inevitably acts at the surface of the peg, and the conditions for glide can only be satisfied when the total reaction from the peg forms a tangent to a circle concentric with the surface of the peg and having a radius* depending on the coefficient of lateral friction and on the radius of curvature of the peg. These conditions are satisfied in Fig. 10,† where the total ventral friction ($Fva+Fvp$) is tangential to the frictional circle at g; the centre of pressure between the snake's body and the peg lies at p, whilst the total reaction (S) from the peg is equal but opposite to ($Fva+Fvp$).

Since the forward glide is dependent on the total ventral friction forming a tangent to the friction circle of the peg, it is of interest to consider the extent to which this fundamental requirement can be satisfied by appropriate muscular activity on the part of the animal. So far, no experimental data are available, but it seems reasonable to suppose that the animal can control the line of action of the *total* ventral friction by bracing its body against static lateral friction between the body and the ground. This principle is illustrated by Fig. 10 (iv) where the line of action of the longitudinal friction passes through the centre of the peg; by bracing an adequate length of its body against static lateral friction, the total ventral friction has become tangential to the friction circle of the peg.

THE PATTERN OF MUSCULAR EFFORT EXERTED BY A GLIDING SNAKE

If the points of application, directions and magnitudes of all the external forces acting against the body of the animal can be determined, it is possible to deduce the pattern of muscular activity involved; at any instant, the muscles acting uniaxially about any vertebral joint must develop sufficient tension to produce about the centre of rotation of the joint a moment (over and above that required to balance the effect of antagonistic muscles) equal but opposite to the sum of the moments exerted by the relevant external forces. Any redistribution of muscular effort automatically involves a redistribution of the pressures exerted by the animal against its external environment. At the same time, however, no muscle—however great may be its tension—can supply useful propulsive energy unless the muscle shortens as progression proceeds.

Applying these principles to a snake gliding through a zigzag channel composed of two rectangular bends (Fig. 8 (i)),‡ two groups of muscles shorten as each region of the body passes a bend. On approaching a bend to the right, the muscles of the right side ($7r$ in Fig. 8 (i)) shorten as the region of the body concerned enters the bend, whilst those of the left side ($7l$) are stretched; on leaving the bend the muscles ($5l$) of the left side shorten whilst those of the right side resume their normal length; when

* If the radius of the peg is r, that of the 'circle of friction' is $r \sin \theta$, where θ is the angle whose tangent is equal to the coefficient of lateral friction (μ). See Fig. 10, Folder III.

† See Folder III.

‡ See Folder I.

passing a bend to the left these changes in length are reversed. As the snake glides forward through the channel shown in Fig. 8 (i), four groups of muscles shorten, viz. 5*l*, 7*r*, 13*r* and 15*l*, and since a muscle can only perform useful external work when it shortens in length, the energy for propulsion can, theoretically, be derived from one or more of these four groups, two of which are located on the left side of the body and two on the right side. In order to determine which of these groups act as prime movers for the animal's progression, it is necessary to consider the distribution of external pressures (against the walls of the channel) which arises in response to activity in each of the four groups of muscles. For this purpose the snake's body can be represented by three units (I–III) as in Fig. 8 (ii). If the muscles (Mr_1) on the inner side of the leading bend develop tension, the region (I) of the body anterior to the hinge *J* is braced against wall B; similarly, if the muscles (Mr_2) on the right of hinge *K* shorten, the posterior unit (III) is braced against wall F. On the other hand, if the muscles on the left side of the body (e.g. Ml_1 and Ml_2) develop tension, sections I and III are braced against walls A and E respectively. The experimental results show clearly that the animal exerts its effort against walls B and F only, and consequently the muscles of the right side are acting as the prime movers for propulsion, whereas those of the left side can be regarded as responsible for the control of the animal's shape and for guiding the body smoothly along the floor of the channel.

The relative tension of the muscles operating about each vertebral joint as determined from the sum of the moments exerted by the external forces is shown in Fig. 8 (iii). The maximum tension is developed by the muscles opposite the centre of pressure (*b*) of the force exerted against wall D of the channel; the whole of the propulsive energy is derived from the muscles Mr_1 and Mr_2, the former group shortening under decreasing tension and the latter shortening under increasing tension. As explained in the Appendix (p. 365), a channel containing two rectangular bends represents the basic requirements for a smooth glide, for it provides three effective *points d'appui* against which the muscles can exert effective leverage. The muscles must develop tension on the side of the body towards which the animal turns when entering the second bend of the channel (i.e. the bend lying nearest to the head of the animal); in every case, the animal exerts pressure against the 'posterior' walls of the channel.

When a snake is gliding round three bends (Fig. 9 (ii)),* it develops its effort against four *points d'appui* (*a*, b_2, *e* and *f*) instead of against three (as in Fig. 9 (i)), all four being situated on the posterior walls (F, D, B and H). The sum of the moments of the external forces about each joint shows that the muscles on the 'inner' (i.e. left) side of the third bend have developed tension. Similarly, when negotiating four bends as in Fig. 9 (iii) the snake utilizes five *points d'appui*, again, all situated on the posterior walls (F, D, B, H and K), and in this case the sum of the moments of the external forces shows that the muscles on the 'inner' (i.e. right side) of the fourth bend are under tension.

* See Folder II.

The general mechanism of propulsion through a zigzag channel containing several bends can be summarized by noting that, as the animal moves into a new section of the channel, the muscles shorten on the side of the body towards which the head turns to negotiate the bend, and at the same time these muscles develop tension against the resistance offered by the 'inner' or more 'posterior' wall of the channel. The active muscles lie on the inner side of each bend; the only exceptions are those situated in the most posterior bend. From a physiological point of view the progress of the animal can be visualized in terms of unilateral waves of muscular posture and tension passing alternately down each side of the body. The form of the waves is determined by the form of the channel, whereas the distribution of muscular tension is, within certain limits, under the control of the animal; for any particular instance, it can be determined by precise observation of the distribution of the forces exerted against the walls of the channel. The distribution of the external forces shown in Fig. 9 is in accordance with the experimental facts; at the same time their magnitude and points of application have been arbitrarily adjusted to yield a state of dynamic equilibrium. The figures should not therefore be regarded as more than a diagrammatic illustration of the general mechanism involved. A more precise relationship between the muscular effort and distribution of external forces involves a consideration of the vertebral column as a series of levers and is given in the Appendix.

THE PATTERN OF MUSCULAR EFFORT WHEN GLIDING PAST A SERIES OF RIGID PEGS

The muscular effort required to glide past a series of rigid pegs is fundamentally similar to that required to glide through a zigzag channel; it is, however, less easy to obtain a complete picture of all the external forces acting against the body and therefore less easy to deduce the underlying pattern of muscular effort.

Fig. 10* shows, diagrammatically, the distribution of muscle tension (as derived from the sum of the moments of external forces) of a snake gliding past the surface of a single peg; the strain falls on the axial muscles lying on the side of the animal which is in contact with the peg. The energy for propulsion is derived from two groups of muscles; those which shorten between the points *c* and *d*, thus drawing the posterior region of the body forwards, and those which shorten between *e* and *f*, thus pushing the anterior end of the body forwards. As the animal glides forward the reaction from the peg remains unchanged, but the effort of the muscles posterior to *p* decreases, whereas that of the muscles anterior to *p* is increased (see Fig. 10 (ii) and (iii)).

The muscular effort necessary to glide past two pegs is shown in Fig. 11. As the snake applies its body to the second peg, the muscles on the side of the body in contact with this peg develop tension, but this can only happen if the snake simultaneously alters the pattern of effort being exerted by the group of muscles which had previously been exerting their effort against the first peg. The nature of this change depends partly on the position of the two pegs relative to the line of action of the

* For Figs. 10–12 see Folder III.

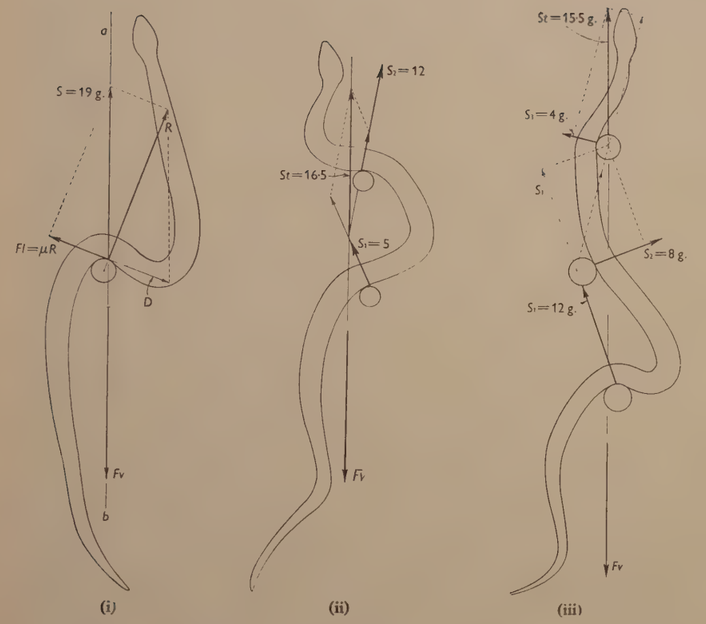

Fig. 6. Tracings from cinematograph records showing the form of the body and the external forces in grams operating against the body of a snake when gliding past one, two and three pendulums. In (i), the reaction (R) normal to the surface of the pendulum has two components D and S; D is equal but opposite to the lateral friction ($Fl = \mu R$) of the pendulum, while S is equal but opposite to the ventral friction (Fv) acting along ab. In other words, the total reaction (S) from the pendulum is equal but opposite to the ventral friction. In (ii), the resultant (St) of the reactions (S_1 and S_2) from the two pegs is equal but opposite to the total ventral friction (Fv). Similarly, in (iii), the resultant of S_1, S_2 and S_3 is equal but opposite to Fv.

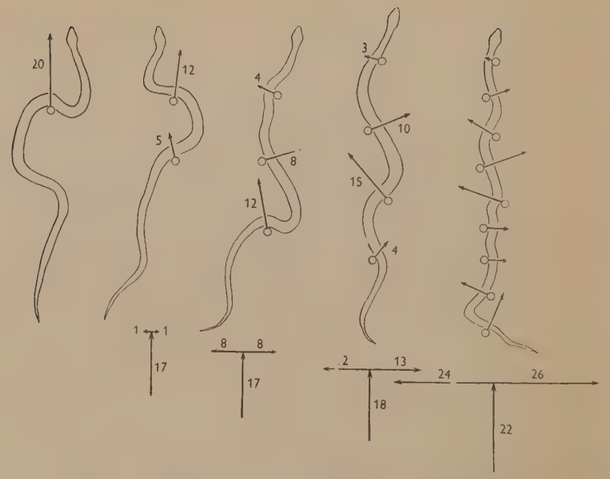

Fig. 7. Records from cinematograph data showing the magnitude in grams and direction of the forces exerted against a series of pendulums. The total forward and lateral forces are shown by the lines drawn beneath each record.

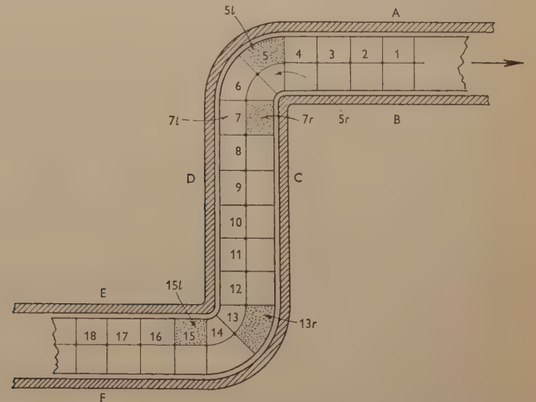

Fig. 8. (i) Diagram showing changes in length of the muscles when a snake is gliding past rectangular bends. *On entering a bend* the muscles on the inner side of the curve shorten whilst those on the outer side are stretched, e.g. the muscles of the right side shorten when a segment moves from position 7 to position 6; also, those of the left side when moving from position 15 to 14. The muscles of the left side are stretched when moving from position 7 to 6, and those of the right side on moving from position 15 to 14. *On leaving a bend* the muscles of the outer side of the bend shorten (5*l* and 13*r*), whilst those of the inner side (5*r* and 13*l*) are stretched.

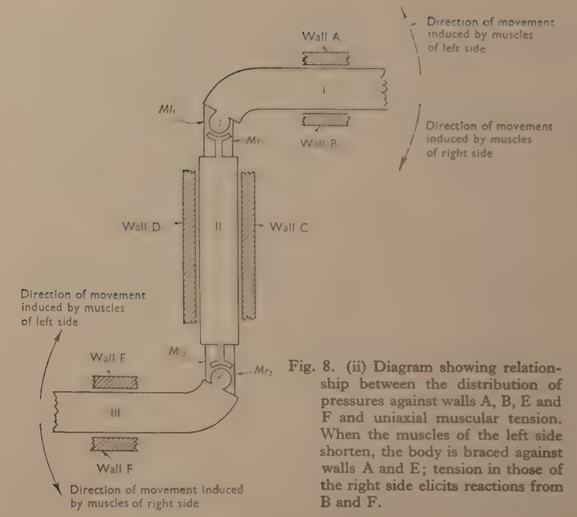

Fig. 8. (ii) Diagram showing relationship between the distribution of pressures against walls A, B, E and F and uniaxial muscular tension. When the muscles of the left side shorten, the body is braced against walls A and E; tension in those of the right side elicits reactions from B and F.

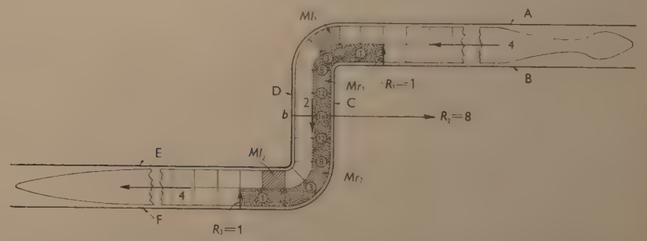

Fig. 8. (iii) Diagram illustrating the general pattern of muscular activity when gliding past two rectangular bends; the ventral friction is shown by the thick arrows drawn along the longitudinal axis of the snake. The walls of the channel are assumed to be perfectly smooth so that their reactions (R_1, R_2 and R_3) act normally to their surfaces. As the snake glides forward the muscles (Mr_1 and Ml_4) of the side of the body lying on the inner side of each bend shorten as the bend is approached, whilst those (Ml_3 and Mr_2) on the outer side of the bend shorten as the bend is being passed. Experimental evidence shows that pressure is applied to the walls B, D and F only; these pressures can only be produced by tension in the muscles of the right side, i.e. the side towards which the animal is turning, in order to negotiate the second or more anterior bend. The tension in the muscles must be such as to produce a bending moment about each vertebral joint equal but opposite to that of the external forces; these moments are shown by the figures in the small circles opposite the various joints.

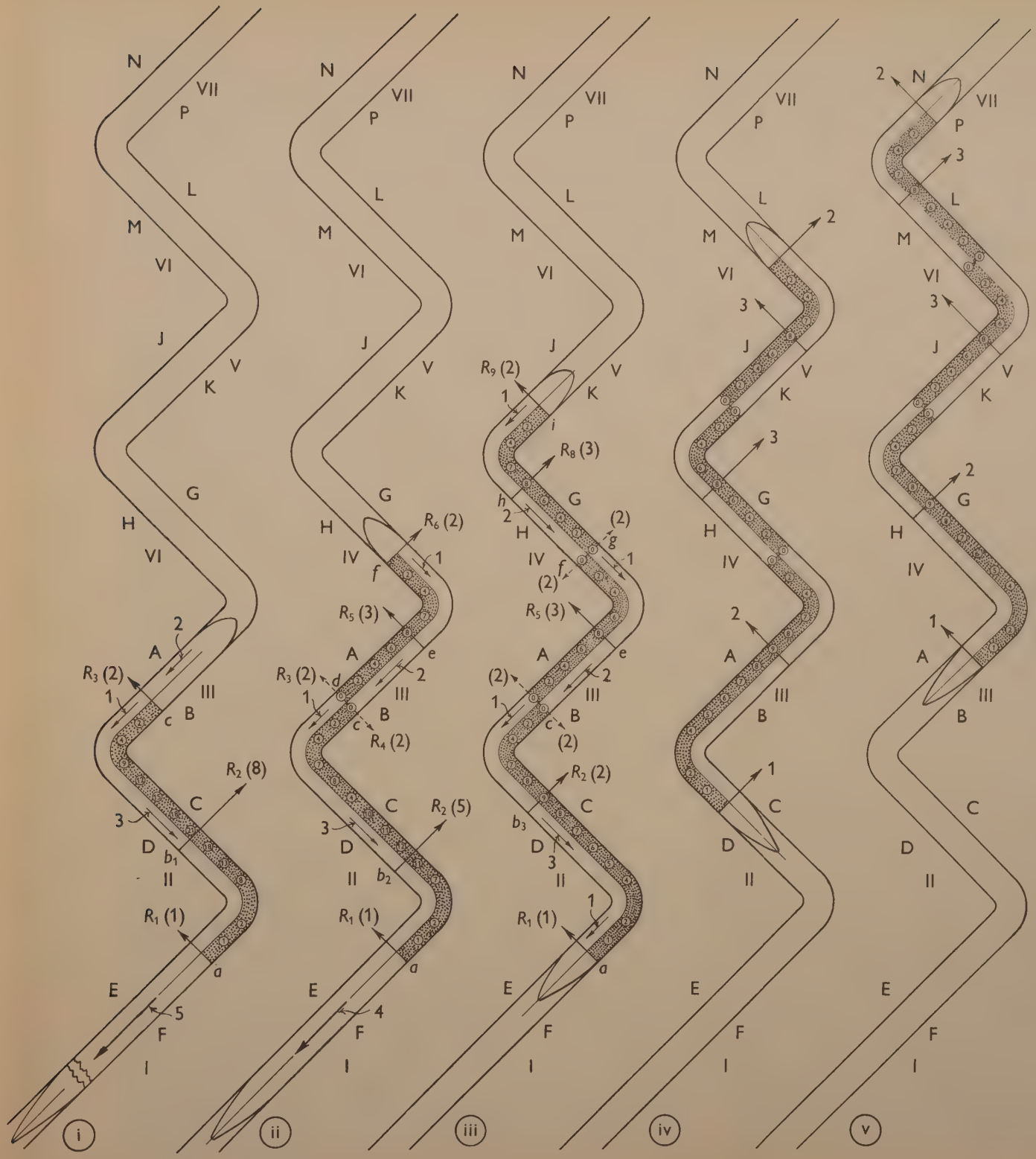

Fig. 9. Diagram illustrating the general pattern of muscular effort of a snake when gliding through a close-fitting rectangular channel. In (i) the reactions acting normally to the walls of the channel at a, b_1 and c yield a resultant equal but opposite to that of the ventral friction of the body; the walls of the channel are, for the sake of simplicity, assumed to be perfectly smooth. The muscles of the right side of the body are under tension. In (ii) the head has moved into section IV and the animal is exerting a pressure against the wall at f; this involves activity of the muscles of the left side; these muscles exert their effort not only at f, but also at e and against the pull of the muscles which previously had exerted a pressure at c. In (iii) a further propulsive unit, operated by the muscles of the right side, develops as soon as the animal begins to exert pressure at i. Similarly, on passing into section VI, the muscles of the left anterior side exert their pressure against wall M (iv); on passing into section VII (v) the muscles of the right side exert their effort against wall P. R_1 (1)...R_9 (2) denote the reactions from the walls of the channel, their relative magnitude being shown in brackets. The magnitude of the ventral friction in each section is shown by the arrows parallel to the long axis of the section. The side of the body where muscles are under tension is shaded; the moments of the muscles about successive vertebral joints are shown in circles.

ventral friction, but it is partly under the control of the animal; it cannot be fully determined without more precise information concerning the points of application o the pressure against the two pegs than is at present available.

When a snake is gliding past a series of pegs the range of effective muscular effort becomes still wider and could only be examined by very extensive experimental analysis. The fundamental nature of the effort can, however, be visualized from Fig. 12, in which the resultant ventral friction is assumed to be distributed as in Fig. 12 (i), and where the points of application of the pressures exerted by the pegs are assumed to be known. When using pegs 1–3 only, as in Fig. 12 (i), the strain falls exclusively on the side of the body towards which the head has turned to gain contact with the most anterior of the three pegs. Thus, in Fig. 12 (i), the strain is on the right side, just as in Fig. 9 (i); substantially the same pattern of muscular effort persists until the anterior end of the animal begins to glide past the surface of peg 4 (Fig. 12 (ii)); at this point the muscles of the left anterior region of the body begin to develop tension. As soon as pressure is exerted against peg 4 (Fig. 12 (iii)), the wave of activity in the muscles of the left side spreads backwards, and this process continues so long as the pressure against peg 4 is increased. As the snake glides forwards to gain contact with peg 5, the muscles of the right anterior side develop tension whilst those of the left anterior side relax (Fig. 12 (iv)). When the tension in the right anterior muscles increases still further, pressure is exerted against peg 5 (Fig. 12 (v)), and the wave of activity on the right side spreads farther backwards along the body. The progression of waves of uniaxial tension alternately down the left and right sides of the animal is illustrated by Fig. 12 (ii–v).

The fundamental principle of peg gliding is clearly the same as that of gliding through a zigzag tube, but the precise pattern of the muscular effort is much more variable. In physiological terms, the glide can be regarded as due to unilateral postural and tensional waves passing alternately down each side of the body. The form of the postural wave is determined by the position of the pegs; the form of the tensional waves is, to a considerable extent, under the control of the animal, and in nature may reasonably be expected to be such as to distribute the total load equitably between the total available musculature.

THE FORCES EXERTED DURING EXTERNAL RESTRAINT

The forces exerted by a freely gliding snake represent only a fraction of those developed when the animal is under restraint. If an isometric lever is attached to the animal at a point immediately posterior to the region which is gliding past the surface of two rigid pegs, the snake continues to glide until the tension of the lever rises to two or three times the weight of the animal (Fig. 13 (i)), after which the tension slowly declines, exhibiting as it does a variable amount of fluctuation. Even greater forces can be exerted if the snake is subjected to a backward pull sufficient to make the animal slip backwards past the pegs; under such circumstances the animal can exert a tension equal to four or five times its own weight. These responses are almost certainly of proprioceptor origin.

Instead of attaching the string from the isometric lever to a point close behind the

region of the body in contact with the pegs, it can be attached to the tail of the animal. In this case a well-marked rhythmical response is superimposed on the sustained pull against the pegs (see Fig. 13 (ii)). The rhythmical response is due to the action of the axial muscles lying posteriorly to the pegs; these muscles throw the

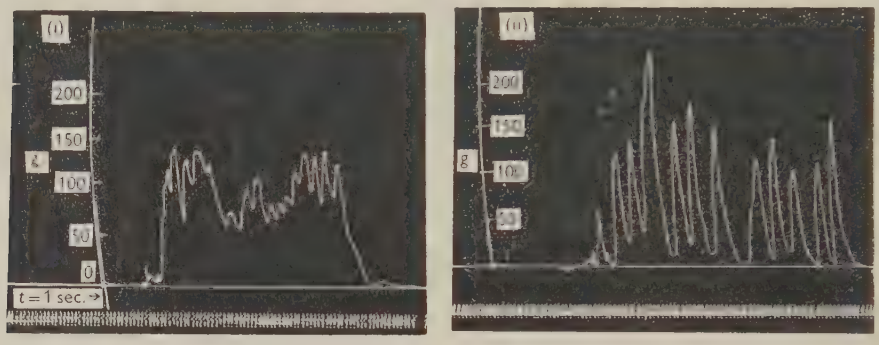

Fig. 13. Tensions set up by a grass snake (83 g.) under restraint. (i) The snake gliding past two rigid pegs is restrained by a string attached a short distance behind the last peg. Fluctuating muscular tension is developed and is maintained for a considerable period. (ii) When the restraining force is applied to the tip of the tail the development of rhythmic tensions is associated with an undulatory pattern of small wave-length and amplitude which spreads in a posterior direction over the body.

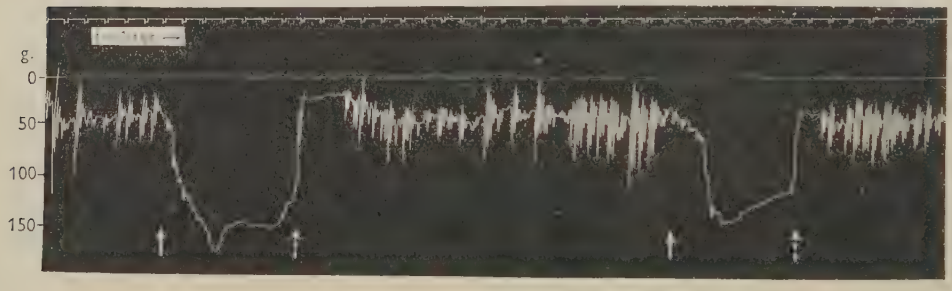

Fig. 14. Tensions recorded from the tip of the tail of a swimming grass snake (67 cm.). At ↑ the body of the snake was allowed to engage against two rigid pegs. This was accompanied by cessation of swimming, while the pull which the snake exerted against the pegs was more than twice its body weight. At ↑ the pegs were removed and the snake resumed swimming.

posterior part of the body into a series of undulations of short wave-length and amplitude, and as the undulations develop the total tension rises. As the undulations die away the tension falls; this process is repeated several times.

If an isometric lever is attached to the tail of a snake, which is otherwise freely immersed in water, the animal swims actively and its body exhibits characteristic undulations of considerable amplitude and wave-length. If one or more rigid objects are then brought gently into contact with the central region of the body, the snake engages with their surfaces and exerts a persistent pull against the lever (Fig. 14), although the region of the body anterior to the points of contact with the pegs may continue to swim actively. On removing the pegs the whole body resumes its

normal swimming movements. These responses recall those of a quadruped, such as a toad, in which diagonal ambulatory movements replace bilateral swimming as soon as the limbs can engage with the ground and elicit an adequate reaction (Gray & Lissmann, 1946). Similarly, the swimming movements exhibited by the front end of a snake whilst its posterior region is pulling against rigid pegs recalls the behaviour of a nemertine under comparable circumstances (Lissmann, vide Gray, 1939).

APPENDIX

The relationship between uniaxial muscular tension and the pressures exerted against external resistances

Although the general patterns of muscular effort can be deduced from an accurate determination of the pressures exerted by the snake against the walls of a zigzag channel, it is perhaps of greater functional interest to consider the converse problem of how the tension of the muscles results in a forward thrust capable of driving the animal forward; thus, in Fig. 8 the tension of the muscles of the right side must be applied in such a way as to yield a resultant external force equal but opposite to the total ventral friction ($F_1 + F_2 + F_3$ (Fig. 15 (i)).*

The relationship of the propulsive force to the tension of the muscles can be visualized by means of two simple but important mechanical principles: (i) When a muscle develops tension across a vertebral hinge, it operates both regions of the body as levers; it applies equal but opposite couples to the structures lying on each side of the hinge, the other arms of the couples being due to compression forces acting across the hinge (see Gray, 1946). (ii) The mechanical effect of a couple is the same as that of any other couple of equal moment and similar sign acting in the same plane. These principles are applied to a snake when gliding past two rectangular bends by dividing the animal into two regions (*xyj* and *jzu*) through the hinge *j* (Fig. 15 (i)) and considering the effect of shortening the muscles (*Mr*) which operate on the right side of this hinge. For the sake of simplicity, it can be assumed that contact between the body and walls B and F is restricted to the points *a* and *c* respectively. When the muscles (*Mr*) tend to shorten, the body encounters resistances applied by the walls B and F, and the muscles therefore develop tension; a clockwise couple (C_1T_1, Fig. 15 (ii)) is applied to the region *xyj* and an equal but anti-clockwise couple (C_2T_2) to *jzu*. The clockwise couple applied to *xyj* can operate this region of the body as a lever in two ways, (i) by using wall B at *c* as a fulcrum and (ii) by using the hinge *j* as a fulcrum. When operating in the first capacity (Fig. 15 (iii)) the couple C_1T_1 is equivalent to a couple P_1Q_1 applied at *c* and at *j*; the force P_1 exerted against wall B elicits an equal but opposite reaction (R_1), and consequently the resultant force exerted on *xyj* is Q_1 operating at the hinge and forming a tractive force pulling against the frictional resistance (F_2) which acts along the axis *yjz*. On the other hand, when the effort of the muscles is exerted against a fulcrum provided by the hinge at *j*, the clockwise muscular couple exerted on *xyj* is equivalent to the couple G_1H_1 (Fig. 15 (iv)), where G_1 is a propulsive force equal but opposite to the frictional resistance (F_1) acting along the axis *xy*, whilst H_1 is a force acting against the hinge. The combined effect of the muscles on *xyj* is therefore to provide propulsive forces which balance the ventral friction (F_1) along *xy* and contribute towards the force required to overcome F_2. The muscles cannot, however, produce these effects without, at the same time, exerting the force H_1 against the hinge (Fig. 15 (v)) and exerting an anti-clockwise couple (C_2T_2) against the region of the body (*jzu*) behind the hinge. The

* See Folder IV.

anti-clockwise couple also operates *jzu* as a lever in two ways: (i) by using the wall F as a fulcrum at *a*, thereby exerting the couple P_3Q_3 (Fig. 15 (iii)); the force P_3 elicits an equal but opposite reaction (R_3) from wall F, whilst Q_3 constitutes a propulsive force along the axis *zjy*, thereby operating against F_2; (ii) by using a point *t* (Fig. 15 (iv)) as a fulcrum and exerting a couple G_2H_2, where G_2 is a propulsive force equal but opposite to F_3 and where H_2 is a force exerted at *t* and parallel to H_1. The muscles can therefore provide propulsive forces equal but opposite to F_1, F_2 and F_3 if, at the same time, wall D provides a reaction (R_2) equal but opposite to the resultant (P_2) of H_1 and H_2 (Fig. 15 (v)). The above analysis involves two artificial simplifications: (i) it is assumed that the body of the snake is braced against walls B, D and F at three points only, (ii) that the walls of the channel are perfectly smooth. Under natural conditions the distribution of pressure between the body and the walls of the channel is under the control of the animal. By appropriate muscular effort the whole of the force required to overcome F_2 can be transferred to wall B or wall F; this involves a movement of the centre of pressure along wall D. If the walls of the channel are not perfectly smooth the effort of the muscles must be increased in order to overcome the resistance due to lateral friction; otherwise the pattern of effort of the muscles remains essentially unchanged.

A channel containing two rectangular bends represents the basic requirements for a smooth glide, for it provides three effective fulcra (*a*, *b*, and *c* in Fig. 15) against which the muscles can exert their effort, the body of the snake constituting a fundamental propulsive unit consisting of two curved levers operated by the muscles lying on the side of the body towards which the more anterior of the two bends is inclined. Once this basic propulsive unit has been established, additional and somewhat simpler units come into action whenever the snake turns past the third, and each successive, bend in the channel. The nature of these additional units can be visualized from Fig. 16*; the only difference from the unit already described lies in the fact that only the more anterior of the two levers is curved, the more posterior lever being straight. In all cases an additional propulsive unit is operated by the muscles on the inner side of the bend in the channel. Fig. 16 (i) shows the forces exerted by a double-bended right-handed unit against the walls of the channel when the unit is operating by itself, whereas Fig. 16 (iv) shows the reactions from the walls when the original double unit is operating with a new left-hand unit such as that shown in Fig. 16 (ii). Fig. 16 (iv) shows that the muscles of the anterior unit are not exerting a force at *d* against the wall A of the channel but against the muscles of the posterior unit; when the new unit becomes operative, the reaction from wall B moves forwards from *c* to *e* (see Fig. 16 (iv)). The formation of successive propulsive units and their effect on those already in operation is shown in greater detail in Fig. 9. In Fig. 9 (i) two rectangular bends are occupied by the animal, and since the more anterior bend is directed towards the right, the whole of the strain falls on the muscles of the right side; these exert their effort against the walls of sections I, II and III at *a*, b_1 and *c* respectively. In Fig. 9 (ii) the head of the animal has passed the third bend, and since this is directed towards the left, the muscles on the left side encounter resistance when the body is in contact with the left wall of the channel at *f*; at the same time, these muscles encounter resistance at *e* and *d*. As this resistance increases the force exerted against *d* becomes equal but opposite to that exerted by the posterior propulsive unit at *c*, and consequently the reaction from the wall at *c* disappears—whilst shearing forces act across the vertebra at the level *cd*; in other words, at this level the active muscles of the leading left-hand propulsive unit are pulling against those of the posterior right-hand unit and vice versa. In Fig. 9 (iii) the head of the snake has passed the fourth bend into section V and a third propulsive unit, operated by the muscles of the right side, has come into action with its muscles exerting their effort against the walls of

* See Folder IV.

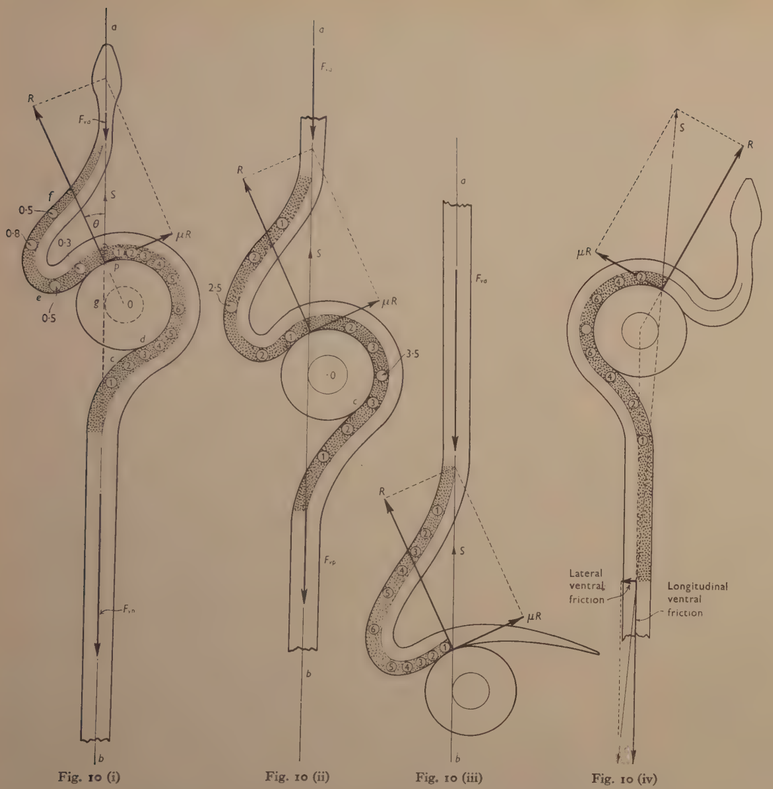

Fig. 10 (i) Fig. 10 (ii) Fig. 10 (iii) Fig. 10 (iv)

Fig. 10. Diagrams illustrating the mechanism of gliding past a single circular peg. If the coefficient of lateral friction between the body and the peg is μ, the total reaction (S) from the peg must form a tangent to a circle, concentric with the surface of the peg, of radius r sin θ, where r is the radius of the peg and θ is the angle whose tangent is μ. The total reaction (S) from the peg must be equal but opposite to the ventral friction ($Fva + Fvp$). The muscles on the side of the body in contact with the peg are under tension, and shorten as they pass from c to d and from e to f, these two groups providing the propulsive energy. As the snake glides forwards the strain on the muscles lying in front of the peg increases, whilst that of those in contact with and posterior to the peg decreases (i–iii). In (iv), the ventral friction passes through the centre of curvature of the peg, and the conditions for glide can only be satisfied if the snake can exert sufficient lateral effort with its tail against transverse lateral ventral friction, to enable the total ventral friction to form a tangent to the circle concentric with the peg.

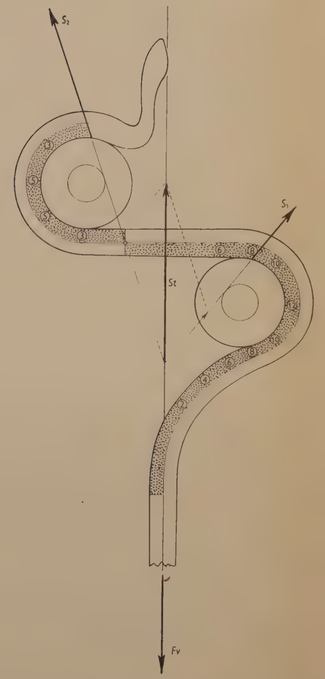

Fig. 11. Diagram illustrating the general pattern of muscular effort when gliding past two rigid pegs. The figure shows the pattern necessary to overcome a ventral frictional force (Fv) acting posteriorly to both pegs; this force is in equilibrium with the reactions (S_1 and S_2) from the pegs. Note that the strain falls on the muscles on the side of the body in contact with the pegs.

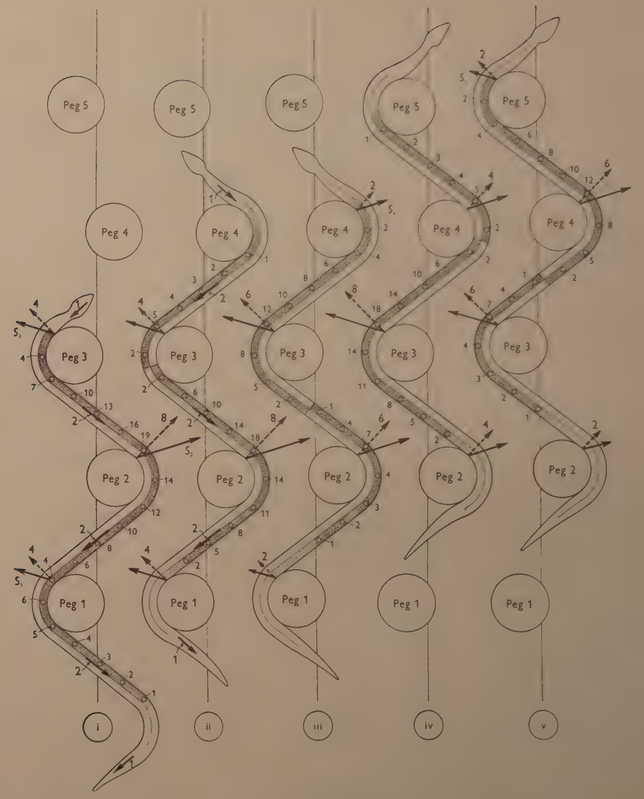

Fig. 12. Diagram illustrating the general type of muscular effort required to glide past a series of rigid pegs. In (i) the snake is exerting its effort against pegs 1–3. The magnitude and direction of the ventral friction are indicated by the length and direction of the arrows lying along the axis of the body. The resultant of all these forces is the same in (i)–(v). The point of application and direction of the total reactions from each peg are shown by the arrows S_1–S_5; the forces acting normally to the surface of the pegs are shown by the dotted arrows. The coefficient of lateral friction is 0·5. All the external forces are in equilibrium. The muscular effort is obtained by taking the moment of all these forces about successive vertebral joints; the value of these moments is shown (approximately) by the figures beside each circle, and recorded on the appropriate side of the body. In (i) the whole of the strain falls on the muscles of the right side, since it is towards this side that the animal has turned to engage its body against peg 3. In (ii) the snake has glided forward turning its left side against peg 4. No pressure is, as yet, applied to peg 4, but the ability to turn towards the left involves shortening of the muscles of that side. In (iii) the muscles of the left side are exerting pressure against peg 4; this involves an increase in the pressure exerted against peg 3 and a reduction of those against pegs 2 and 1. In (iv) the snake has glided forward and has turned to the right to gain contact with peg 5, thus involving activity in the muscles of the right side. Simultaneously, the animal has lost contact with peg 1; the pressure against peg 2 has decreased, whilst the pressures against pegs 3 and 4 have increased. In (v) the snake is exerting pressure against peg 5, and this involves a change in the pressures against pegs 2, 3 and 4. Note the passage of uniaxial muscular waves of tension along the side of the body in (ii)–(v).

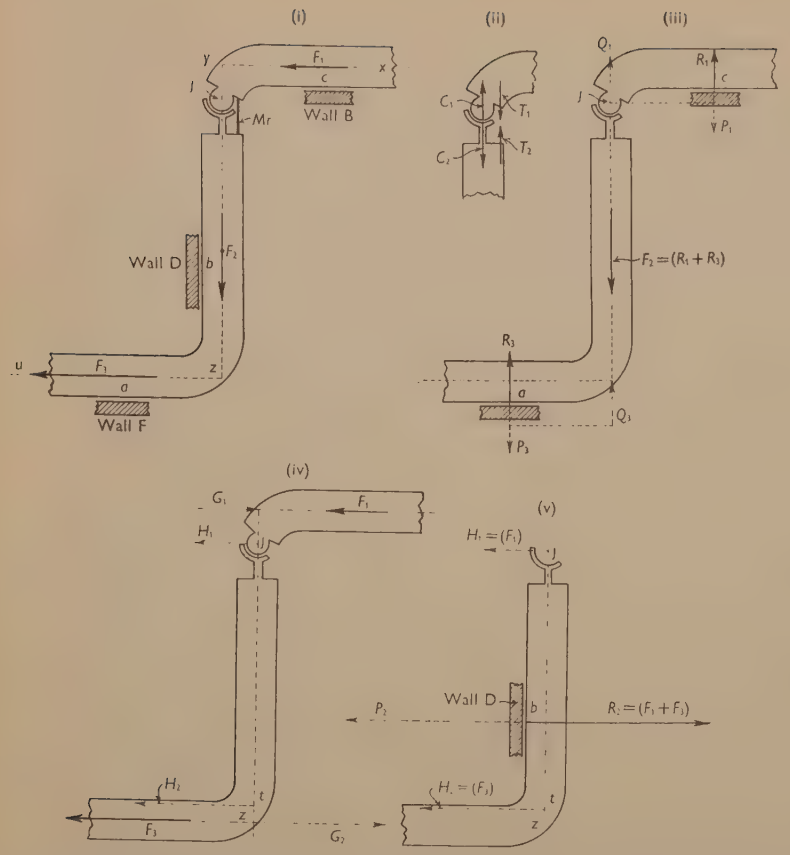

Fig. 15. Diagram illustrating the origin of propulsive forces when the axial muscles act against the walls of a rigid channel containing two rectangular bends, the more anterior of which is directed towards the right side of the animal. The frictional forces restraining forward motion are F_1, F_2 and F_3 acting along the axes xy, yz and zu respectively. The snake is divided into two parts at the hinge j, the muscle (Mr) acting on the right side of the hinge; the snake's body is in contact with the walls B, D and F of the channel, and it is assumed that pressures against B and F can be exerted only at a and c respectively (i).

When the muscles at j develop tension against the restraints imposed by the forces F_1, F_2 and F_3 and by the walls of the channel they exert a clockwise couple (T_1C_1, fig. 15 (ii)) against the section (xyj) of the snake anterior to the hinge, and an equal but anti-clockwise couple (T_2C_2) against the section (jzu) posterior to the hinge. As shown in fig. 15 (iii), the couple T_1C_1 is equivalent to a couple Q_1P_1 having similar direction and equal moment to T_1C_1; P_1 elicits an equal but opposite reaction (R_1) from wall B at c, and consequently the resultant force acting on xyj is Q_1 acting along the axis yz and operating against F_2. Similarly, the anti-clockwise couple T_2C_2 acting on jzu is equivalent to a couple Q_3P_3, in which Q_3 represents a forward thrust along yz, whilst P_3 elicits from wall F a reaction (R_3) equal and opposite to P_3 at a. As shown in (iv) the muscles acting about j can exert their effort against the forces F_1 and F_3 as well as against F_2; in this case the couple acting on xyj is equivalent to G_1H_1, thus providing a forward thrust against F_1, whilst the couple acting on jzu is equivalent to G_2H_2, thus providing a forward pull against F_3. As shown in (v), a force H_1 (equal to F_1) acts against the hinge, and a force H_2 (equal to F_3) acts at t; the resultant of these two forces is a total pressure P_2 exerted against wall D at b. The wall yields a total reaction (R_2) equal but opposite to P_2.

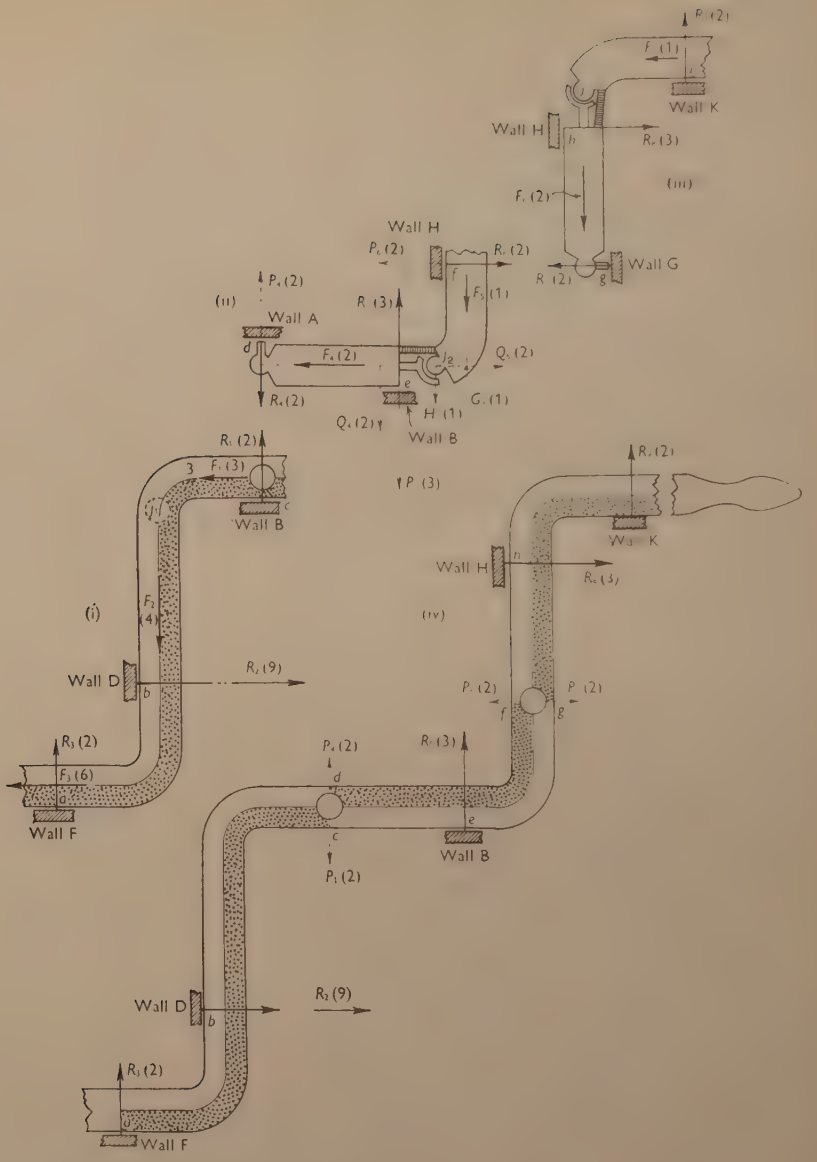

Fig. 16. Diagram illustrating the operation of additional propulsive units when the snake can exert its muscular effort against three or more rectangular bends. R_1–R_9 denote the reactions from the walls of the channel. F_1–F_7 denote the ventral friction between the snake and the floor of the channel. The figures in brackets indicate the relative magnitude of the forces. In (i) the snake is utilizing walls B, D and F as in previous figures; it is propelling itself by means of the muscles of its right side and eliciting a reaction R_1 (2) from wall B at c. (ii) denotes a new anterior unit operating against walls H and B, and consisting of a bent lever fj_2 and a straight lever j_2d. The muscles on the left side of the joint j_2 exert on the two levers couples (P_6Q_6, G_3H_3, P_4Q_4) similar to P_1Q_1, G_1H_1 and G_2H_2 in Fig. 15 (iii) and (iv). The resultant of H_3 and Q_4 is P_5; the latter force elicits a total reaction R_5 from wall B. When this new propulsive unit operates with that shown in (i), the force exerted against the wall A at d is equal but opposite to that exerted by the posterior unit against wall B at c, and therefore there is no reaction from either wall at c or d. (iii) shows a third propulsive unit operating against walls K, H and G. (iv) shows the three units acting together eliciting reactions (R_3, R_2, R_5, R_8 and R_9).

sections V and IV, at *i* and *h*, and against the muscles of the more posterior propulsive unit at the level *fg*. It will be noted in Fig. 9 (iv, v) that the centres of pressure between the body and the walls of the channel are situated towards the anterior end of each channel (just before the next bend in the channel is reached). This is in conformity with the positions actually observed when the snake moves through a channel somewhat wider than its own body and when the snake is made to exert its effort against a wall composed of a series of independently mounted elastic strips of metal; in the latter case the greatest deflexion is observed at points similar to those indicated in Fig. 9 (iv, v).

SUMMARY

1. The coefficient of sliding friction between the ventral or lateral surface of a grass-snake varies from 0·2 to 0·6 according to the nature of the surface over which the animal is moving.

2. The distribution and magnitude of the external forces acting against a moving snake has been determined for (i) an animal moving through a close-fitting zigzag channel, and (ii) an animal gliding past one or more rigid pegs. The pattern of muscular effort required for these two types of motion is described. Forward movement is due to the operation of the vertebral column as a series of levers.

3. For a freely moving snake the propulsive force is of the order of one-third of its total body weight; when motion is artificially restrained a grass-snake can sustain a pull of four or five times its own weight.

4. The circumstances under which a snake can exhibit typical terrestrial locomotion as opposed to the movements exhibited in water are fundamentally similar to those of a quadruped such as a toad.

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RECTILINEAR LOCOMOTION IN A SNAKE (*BOA OCCIDENTALIS*)

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(With Seven Text-figures)

The mechanics of three common modes of locomotion in snakes has been subjected to a detailed analysis (Gray, 1946; Gray & Lissmann, 1950). These three modes are (i) the serpentine or horizontal undulatory movement; (ii) the concertina movement; (iii) the crotaline or side-winding movement. A fourth, less common mode, described as 'rectilinear movement', has aroused considerable interest since Home (1812) published his 'Observations intended to show that the progressive motion of snakes is partly performed by means of ribs'.

During rectilinear locomotion the whole snake moves forward along a straight line. Lateral bending of the body and lateral resistances from the substrate do not form an essential feature of this movement; it is, therefore, quite unlike the three first-mentioned types. A characteristic of all species in which rectilinear movement has been described (e.g. Boidae, Viperidae, *Ophisaurus*) is a strongly developed ventral cutaneous musculature. Most observers are agreed that waves of muscular contraction followed by relaxation pass over this musculature. Wiedemann's (1932) explanation of the mechanics of this movement can be summed up by his term 'snail principle'; according to this author the waves of muscular contraction travel in a postero-anterior direction. Mosauer's (1932) designation 'caterpillar movement' might imply the same direction of travel, but he states that the movement of the locomotory waves is from head tailward.

Antero-posterior movement of a peristaltic wave would suggest an earthworm's rather than a snail's or a caterpillar's mode of pregression. The term 'earthworm principle', however, has been applied by Wiedemann (1932) to the concertina movement. Observations by Wiedemann (1932), Mosauer (1932) and Bogert (1947) suggest that the ribs do not play an active part in rectilinear locomotion.

The differences in kinetics and in neuro-muscular co-ordination between a moving snail and an earthworm are very considerable (see Lissmann, 1945*a*, *b*; Gray & Lissmann, 1938*a*). Moreover, these invertebrates do not possess a system of rigid levers comparable with the limb bones or vertebrae which play an essential role in most, if not all, vertebrate propulsion. It therefore appeared of interest to obtain additional information about the rectilinear type of progression.

The most strongly developed ventral cutaneous musculature occurs in Boidae. I am indebted to the Zoological Society of London for two young specimens of *Boa occidentalis* (57 and 60 cm. long) which were used for the observations reported below.

MOVEMENT DURING RECTILINEAR PROGRESSION

Rectilinear movement in *B. occidentalis* is a slow method of progression often employed when the snake approaches the living prey. It can also be readily elicited by placing the animal on a smooth surface. To obtain an adequate picture of this

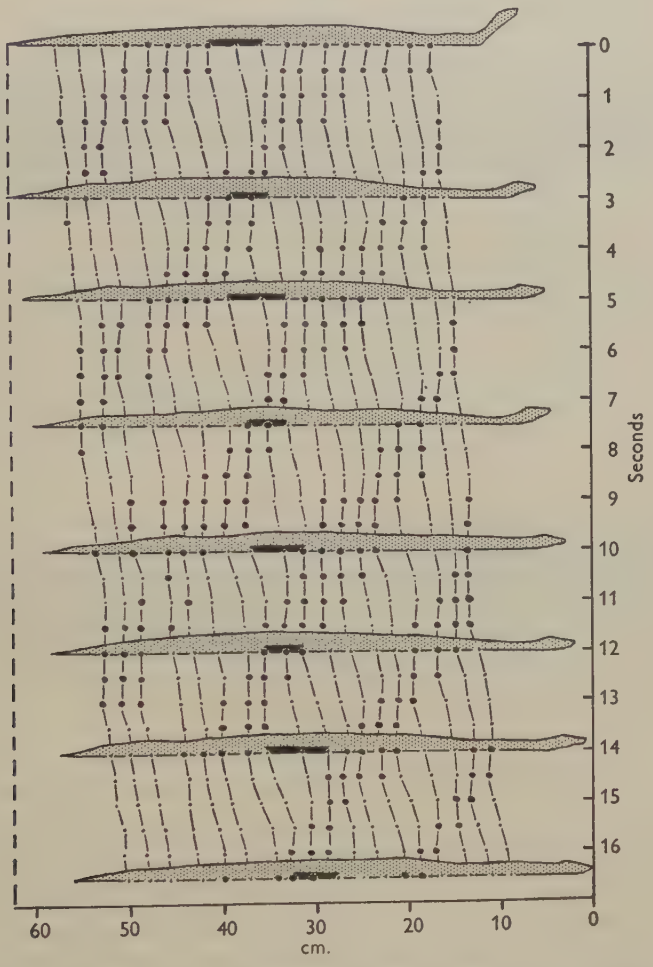

Fig. 1. Rectilinear progression in *Boa occidentalis* (redrawn from film). The vertical lines indicate successive positions of points marked on the ventro-lateral surface. Heavy dots indicate that the points remain stationary relative to the ground or that they exhibit a backward slip; fine dots show the points in forward motion. Note the alternation of shortening and elongation between adjoining points (e.g. the black area in the centre of the body) which passes in form of waves in a posterior direction over the body. At any one time two complete wave-lengths usually extend over the body.

type of movement simultaneous cinematograph pictures were taken of the lateral and ventral aspects of a snake by allowing it to creep over a glass plate and placing an inclined mirror under the glass. In an analysis of the photographs the natural

markings and scales of the *Boa* can be used to advantage; white paint marks were also found useful.

The general nature of the forward motion is shown in Fig. 1, in which the relative locations of eighteen individual points on the ventral lateral surface have been marked in thirty-four successive positions. It will be seen that the distance between

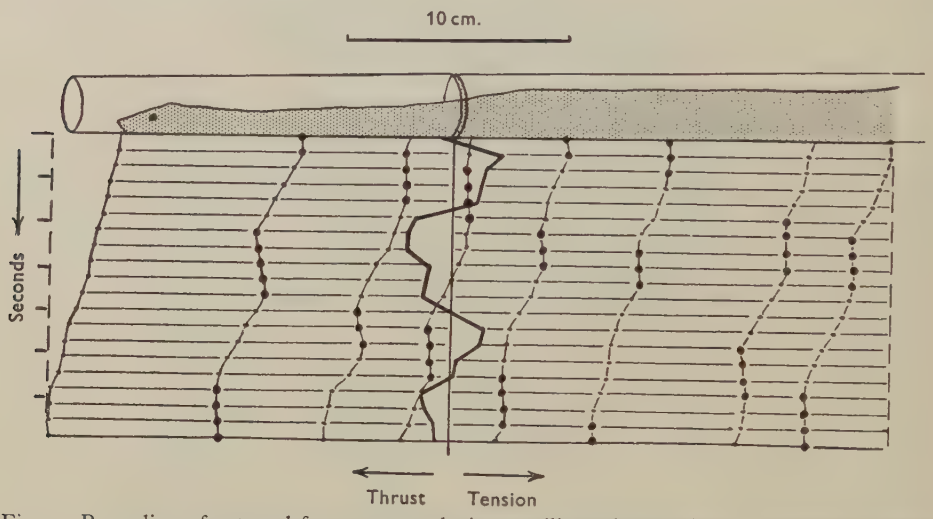

Fig. 2. Recording of external forces set up during rectilinear locomotion. The snake is made to creep through a rigidly fixed glass tube (right) across a narrow gap into a second tube (left) which is suspended by steel springs. The movements of the second tube are magnified by levers and are photographed simultaneously with the movements executed by the snake. Note that the head of the animal moves forward at relatively constant speed, whilst the ventral surface moves forward in 'steps'. Areas of fixation to the ground are indicated by heavy dots. As the areas of fixation move posteriorly over the body and approach the gap, tensions are set up between the two tubes (graph to the right of the vertical line); when the area of fixation has traversed the gap a longitudinal thrust develops (left part of graph).

two points lying close together varies rhythmically in such a manner that waves of alternating longitudinal contraction and elongation appear to pass in a posterior direction over the body. During steady progression two complete waves have been generally observed on the body. As the waves pass along, each point on the ventral surface of the animal is carried forward in rhythmic steps of 2–3 cm. in length. These waves exhibit occasional irregularities and may—as in the earthworm—fail to reach the posterior end of the body.

The similarity between *Boa* and earthworm is emphasized by observations of the external mechanical forces set up during locomotion. The nature of these forces has been recorded by allowing the snake to creep through a rigidly fixed, horizontal glass tube into a second tube which was movably suspended by steel springs. The movement of these springs, indicating tensions and thrusts set up between both tubes, was magnified by levers and was recorded photographically. Fig. 2 shows that a tension is developed as long as an area of fixation of the snake lies anteriorly in the movable tube. When this area of fixation has travelled backwards over the

body and has reached the rigid tube tension changes into a longitudinal thrust—in a manner comparably recorded in the earthworm (Gray & Lissmann, 1938*b*).

There is, however, one significant difference between the two animals: while all parts of the earthworm move forward in 'steps', certain parts of the *Boa*, e.g. the head in Fig. 2, progress at relatively constant speed.

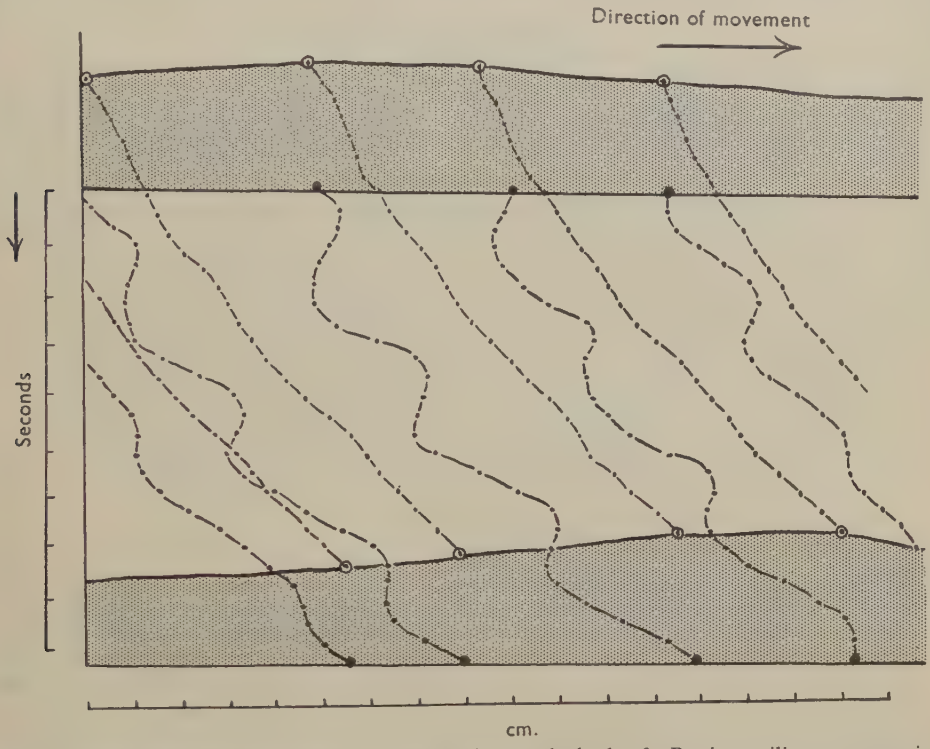

Fig. 3. Forward movement of dorsal and ventral points on the body of a *Boa* in rectilinear progression. The shaded areas represent the lateral view of the middle region of the body. Note that the dorsal points move at relatively constant speed, whilst the ventral points move forward in 'steps' each 2–3 cm. long.

This is shown in greater detail in Fig. 3, which also shows that whereas progression of any point on the ventral side is characterized by periods of forward movement alternating with periods of rest, any point located on the dorsal side moves forward at relatively constant speed and, therefore, all points on the dorsal side retain an equal distance from one another throughout the locomotory cycle. If the muscular waves are regular, this is also true for any two points on the ventral side as long as the distance between them is one complete wave-length or the multiple of one complete wave-length.

Maximal changes in length occur between two points on the ventral surface which lie half a wave-length apart, e.g. point *g* and *o* in Fig. 4B. Provided there is no backward slip, the length of a step is determined by the difference of maximal elongation and maximal shortening between these two points; the speed of the whole animal is the length of a step multiplied by the frequency of waves which

pass over the body. The telescoping of the ventral scales provides a useful measure of the degree of contraction of the ventral musculature. In Fig. 4A the forward movement, relative to the ground, of every fifth ventral scale of the central portion of the body is shown. A clearer picture of the shortening and elongation is obtained for the same region by plotting the movement of the scales relative to a fixed point

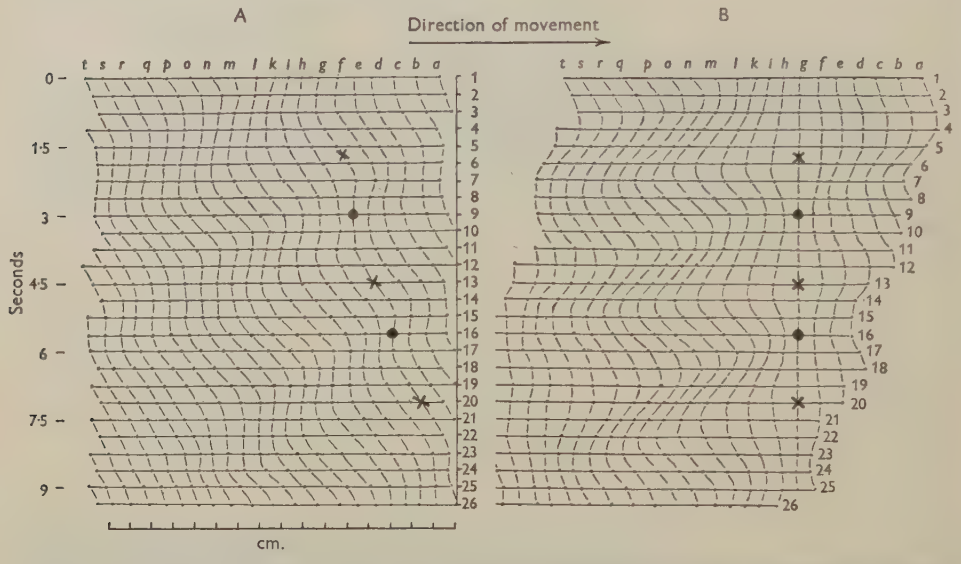

Fig. 4. The points *a*, *b*, *c*, etc., on the horizontal lines indicate the distances between five ventral scales. (A) shows the forward motion relative to the ground of these points; (B) shows the changes in shape on the body during the same period; the distances between the points are plotted relative to point *g*. Note that conditions of maximal extension of the regions adjoining *g* (marked ×) occur at positions 5–6, 13 and 20. In this state the scales glide forward relative to the ground with maximal velocity (× in A). Maximal contraction near point *g* occurs at positions 9 and 16 (marked ●). These positions coincide with the stationary phase in Fig. 4A. Point *o* (in B) is about half a wave-length posterior to point *g*; the difference between its maximal elongation from *g* (positions 7, 14 and 22) and its maximal contraction towards *g* (positions 11 and 18) equals the length of one 'step'.

on the body (point *g* in Fig. 4B). Comparison of Fig. 4A and B shows that as long as a region of the body is maximally contracted it remains stationary relative to the ground, and on a very smooth surface may even slip backwards. This region begins to glide forward with increasing velocity as soon as relaxation sets in and slows down with the onset of the next wave of longitudinal contraction until it comes to rest again in a state of maximal contraction.

Half a wave-length of muscular activity extends over a region in which some 20–30 ribs can be counted on either side. If the average step is about 2 cm. long a change of distance less than 1 mm. between two adjacent tips of the ribs could account for the changes in length observed on the surface during one locomotory cycle. On the other hand, if the movements of the ribs are similar to those of vertical lines drawn on the skin of the animal then any rib should swing through a considerable angle round its base when the adjoining musculature passes from a condition

of extreme contraction to one of extreme elongation. These movements might be expected to be parallel to the movements of vertical lines drawn on the body of a creeping animal as shown in Fig. 5.

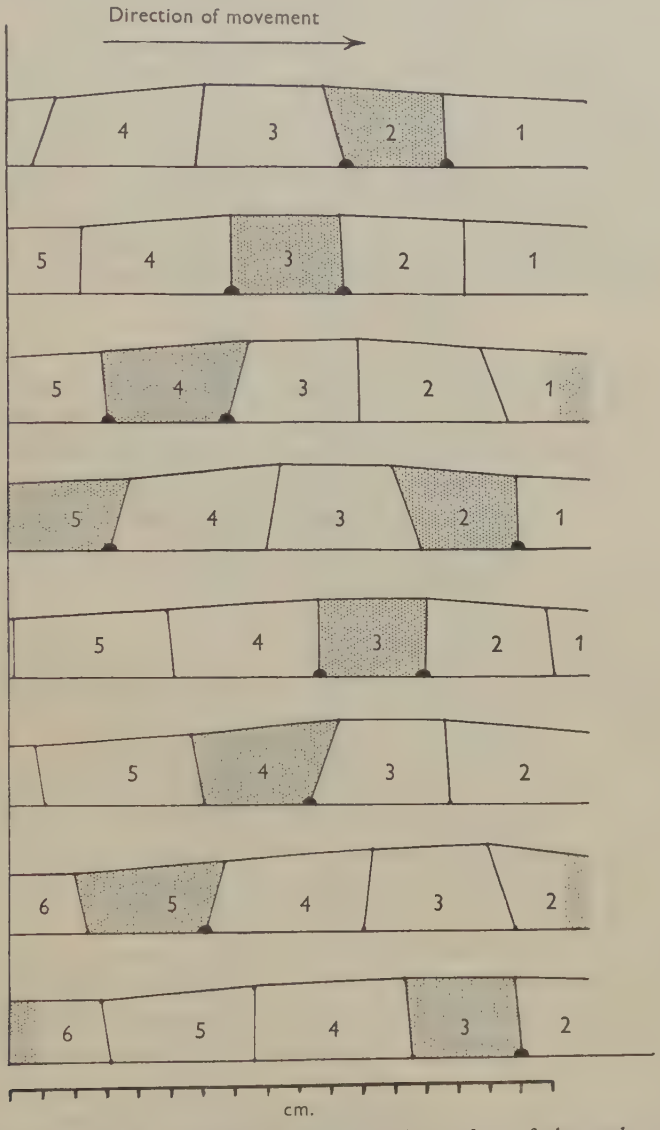

Fig. 5. Forward movement of vertical lines drawn on the surface of the snake during 6 sec. of rectilinear progression. The state of maximal contraction of a region between two lines is indicated by shading; the region posterior to it undergoes contraction whilst the anterior section elongates. Comparison of a region during maximal contraction and maximal elongation shows that the angle which the vertical lines form with the horizontal (vertebral column) varies considerably. Areas of fixation to the ground are marked by heavy ventral dots.

In order to determine whether the tips of the ribs execute any appreciable movement relative to the vertebral column during movements exhibited by the skin,

small lead disks were attached to the ventral lateral surface of a snake and successive X-ray photographs were taken of the various phases of the locomotory cycle.* The movement of the disks relative to the ribs and the vertebral column is given in Fig. 6 for three positions. It will be seen in Fig. 6 (i) and (iii) that the centre of maximal elongation is between the 11th and 26th vertebrae; Fig. 6 (i) also shows the succeeding posterior centre of contraction between the 67th and 77th vertebrae. In Fig. 6 (ii) the states of contraction and elongation are reversed at the same levels. In all figures the tips of the ribs retain accurately the same positions relative to the vertebral column, whereas the lead disks glide past the tips of the ribs; the same surface region, when elongated, extends over six more ribs than it does in a contracted state. This result is in agreement with the observations of Wiedemann (1932), Mosauer (1932) and Bogert (1947) and in contradiction to the 'rib-walking theory'.

DISCUSSION

The picture of the mechanics of rectilinear progression can be elucidated to a certain extent by taking into account the following facts: (1) the ventral surface of the *Boa* moves forward in rhythmic steps, while waves of muscular contraction and relaxation pass in a posterior direction over the ventral cutaneous musculature; (2) the vertebral column moves forward at relatively constant speed; (3) the ribs do not perform any appreciable movement relative to the vertebral column; (4) the vertebral column and the ventral cutaneous musculature are mechanically connected through the ribs and two distinct sets of muscles.

A knowledge of the geometrical arrangement of the different muscles makes it possible to determine their changes in length throughout a locomotory cycle as long as the length of any one of them is known.

Ventral cutaneous musculature. On anatomical grounds this massive muscle can be expected to represent the main source of energy for rectilinear progression. Its changes in length have been described in the previous section, but the mechanism of these changes remains controversial. During movement, each complete locomotory wave can be subdivided diagrammatically into four regions (*A* to *D*, Fig. 7, 1†) according to the state of muscular activity.

(i) An anterior group of ventral cutaneous muscle fibres is in a state of maximal contraction (Fig. 7, 1, *A*) and cannot provide any propulsive energy to the rest of the system, although this region can form a *point d'appui* against which muscles lying posteriorly can exert a tractive force. The fixation to the ground on a smooth surface such as glass can only be due to static friction. On rough ground the free posterior margins of the scales assist in anchoring this area to the substrate.

(ii) Immediately posterior to the region of maximal contraction is a region undergoing contraction (Fig. 7, 1, *B*). This is the only group of ventral cutaneous muscle fibres which are active in a locomotory sense, for when they shorten actively they develop tensions against frictional resistance of the ground. Any point of this contracting region moves forward with decreasing velocity.

* My thanks are due to Mr J. A. Fozzard for his assistance in taking these photographs.

† For Fig. 7 see Folder V.

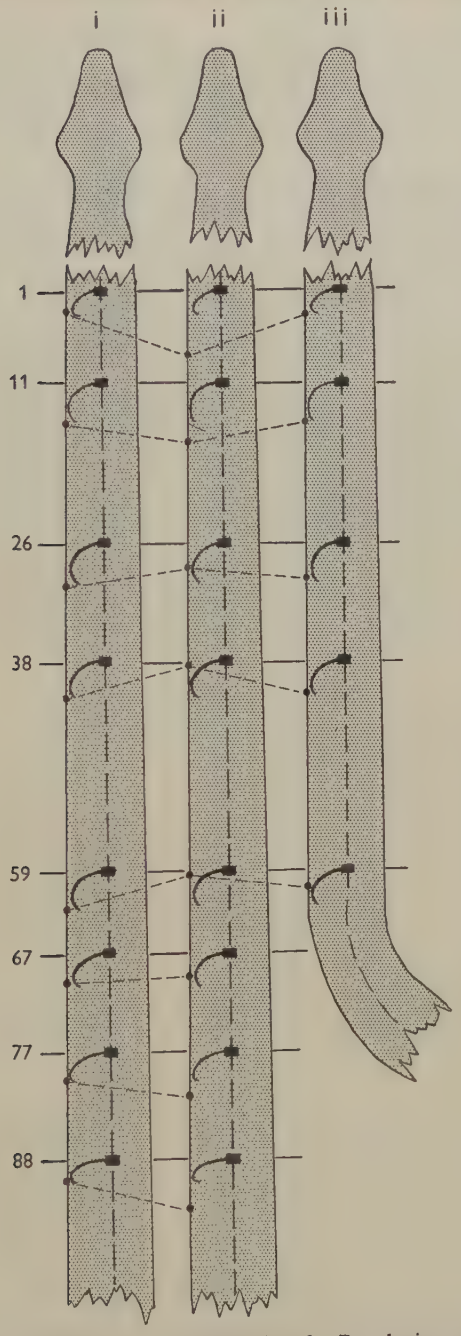

Fig. 6. Analysis of three successive X-ray photographs of a *Boa* during rectilinear progression. The points on the outline of the animal indicate the positions of lead disks attached to the surface. Other lead disks along the median dorsal line mark the vertebrae. The centre of maximal elongation for (i) and (iii) lies between the 11th and 26th vertebrae, for (ii) between the 67th and 77th. Centres of contraction are between the 11th and 26th vertebrae in the case of (ii), and between the 67th and 77th in the case of (i). Note that the lead disks on the ventro-lateral surface are displaced relative to the vertebral column, while the position of the tips of the ribs does not change.

(iii) The next posterior region (Fig. 7, 1, *C*) comprises muscle fibres of maximal elongation moving forward relative to the ground with maximal velocity. The velocity of this region is greater than that of the vertebral column; it does not, however, contribute any energy for its own forward motion or for that of any adjoining part.

(iv) Fig. 7, 1, *D* represents a region in which the muscle fibres elongate, each point moving forward with increasing velocity. As long as this region lies posterior to a *point d'appui* the elongation of its fibres offers no theoretical difficulty, for elongation could be achieved by passive stretch from an anterior contracting region (*B*) acting against friction. However, if within the cycle of activity the phase of maximal contraction is reached at the most anterior part of the body (e.g. Fig. 7, 1 and 5) there would be no anterior cutaneous muscle fibres which could contribute to an elongation by pulling against an anterior point which is fixed to the ground.

Wiedemann (1932) assumed that elongation of the contracted longitudinal fibres is accomplished by passive relaxation of the elastic connective tissue. This would mean that during longitudinal contraction energy is being stored in the connective tissue and becomes effective as soon as tension in the ventral musculature ceases. Wiedemann observed an elongation of the body of a decapitated snake after release from longitudinal compression. In the absence of more precise data it is impossible to decide how far such an elastic recoil is sufficient in extent, speed and power to elongate, against friction, the ventral cutaneous musculature; further, this explanation does not cover the fact that part at least of the extension may be due to the passive stretch exerted by the vertebral column which is moving forward continuously.

An alternative and more active mechanism of elongation of the ventral cutaneous musculature is suggested by the distribution of the muscles uniting the ventral surface of the body to the ribs. These muscles form two groups (Buffa, 1905): (i) *M. costo-cutanei superiores* running from each rib to a more posterior region of the ventral skin; (ii) *M. costo-cutanei inferiores* running from the ribs to more anterior regions of the ventral skin (Fig. 7). If these muscles maintain constant length during the whole locomotory cycle they must act as a rigid system whereby the forces exerted by the ventral musculature are transmitted to the dorsal regions of the body and vice versa. If the elongation of anteriorly contracted fibres of the ventral cutaneous musculature is to be effected at the required rate by means of a rigid connexion with the vertebral column, there can be only one point at which the movement of the cutaneous musculature can be transmitted to the vertebral column by a non-extensile element. This point must be located exactly one wave-length behind the anterior end of the ventral cutaneous musculature. Such a rigid attachment would, however, mean that the vertebral column would have to move forward in the same rhythmic manner as its point of attachment at the ventral surface and not at constant speed. Since the vertebral column does move forward at constant speed, and since it has muscular attachments with the ventral skin at many points along the entire locomotory wave, the muscles forming this connexion must undergo cyclic changes in length as is diagrammatically shown in Fig. 7, 1–7.

M. costo-cutanei superiores. The changes in length of these muscles in co-ordination

with the activity of the ventral cutaneous musculature can be followed from Fig. 7, 1–7. The anterior end of the ventral surface presents the clearest mechanical conditions. In Fig. 7, 1 the anterior region *A* of the ventral cutaneous musculature is maximally contracted and is fixed to the ground while the vertebral column and ribs are travelling forward at constant speed. During the transition from Fig. 7, 1 to 2 the posterior margin of region *A* remains stationary while the anterior margin begins to move forward with increasing velocity and must, therefore, overcome longitudinal ventral friction. The force required to overcome this friction and to stretch the muscles can be derived from the longitudinal thrust of the vertebral column which is propelled forward through the activity of more posteriorly situated sections of the musculature. This forward thrust (recorded in Fig. 2) is transmitted to the anterior margin of region *A* of the ventral cutaneous musculature via anterior ribs and *M. costo-cutanei superiores*. Since the anterior margin of the ventral cutaneous musculature begins to accelerate from rest while the vertebral column travels forward at constant speed, the *M. costo-cutanei superiores* must at first be stretched passively while developing sufficient tension to overcome ventral friction. The rate of stretch decreases, presumably with increasing tension, until the anterior margin of region *A* of the ventral cutaneous musculature has attained a speed equal to that of the vertebral column; at that point the length of the *M. costo-cutanei superiores* remains constant.

However, throughout each complete locomotory cycle the average speed of the vertebral column and of the ventral surface must be the same. Since the ventral surface remains stationary during part of the cycle its speed must, at times, exceed that of the vertebral column. The acceleration of the ventral surface beyond the speed of the vertebral column can only be achieved through an active shortening of the *M. costo-cutanei superiores* (Fig. 7, 2–3). Since these muscles shorten at the same time as developing tension against the frictional resistance of the anterior region they must contribute propulsive energy to the whole system. The phase of active contraction sets in shortly after the point of cutaneous attachment of this muscle has begun to glide forward (i.e. between positions 1 and 2 in Fig. 7); it extends until this point has passed its maximal velocity and begins to slow down (i.e. between positions 3 and 4 in Fig. 7).

Conversely, as soon as the point of the ventral surface to which the *M. costo-cutanei superiores* are connected has slowed down to a speed below that of the vertebral column, these muscles will be passively elongated through the forward movement of the vertebral column; this occurs before the point of the ventral surface becomes stationary relative to the ground, e.g. at the anterior end between positions 3 and 4 in Fig. 7. This elongation will continue throughout the period of fixation to the ground (positions 4 and 5, Fig. 7). During this period tension in the *M. costo-cutanei superiores* could only have the effect of resisting the forward motion of the vertebral column, and it seems likely that the muscles remain inactive in this phase. Elongation ceases between positions 5 and 6 in Fig. 7, as soon as the velocity on the ventral attachment again equals that of the vertebral column.

This scheme of rhythmic changes in length and tension has been arrived at by

considering the anterior end of the animal. If the regularity of movement is to be maintained and if the muscles are not to slacken between their points of attachment, similar changes in length must occur throughout the whole length of the animal. It is not equally certain that comparable tensional changes also occur, because additional forces for elongation and propulsion may become available from the contracting regions of the ventral cutaneous musculature as long as they are situated posterior to a *point d'appui*.

Apart from the horizontal forwardly directed force of the *M. costo-cutanei superiores*, it may be assumed that, by virtue of their more dorsal attachment on the ribs, contraction in these muscles may tend to elevate or to reduce the pressure on the ground of those parts which are gliding forward (Fig. 7), thus reducing their friction. This would inevitably increase the pressure and efficiency of the areas of fixation.

M. costo-cutanei inferiores. The picture of rectilinear movement derived so far is based on the assumption that the vertebral column is travelling forward at constant speed, without considering the forces which propel it forward. The position of the *M. costo-cutanei inferiores* suggests (Fig. 7) that these muscles transmit to the vertebral column the propulsive force exerted by the ventral cutaneous muscles. It seems reasonable to suppose that the length of these muscles changes rhythmically in a manner similar to that of the *M. costo-cutanei superiores*, except that the *M. costo-cutanei inferiores* must shorten wherever their point of attachment to the skin travels forward more slowly than the vertebral column (e.g. at the anterior end in Fig. 7 this period must start between positions 3 and 4, and it must end between positions 5 and 6). Only in this phase can the *M. costo-cutanei inferiores* directly contribute a propulsive thrust to the vertebral column. It is interesting to note that this contraction begins as a *point d'appui* approaches from the anterior end the cutaneous attachment of this muscle; it persists throughout the period of fixation to the ground and includes the early stages of acceleration.

Elongation of the *M. costo-cutanei inferiores* takes place whenever the point of cutaneous attachment travels faster than the vertebral column, i.e. at the anterior end beginning between positions 1 and 2 and ending between positions 3 and 4 in Fig. 7. If these muscles develop tension during the phase of elongation they would act directly against the propulsive phase of the *M. costo-cutanei superiores* and resist the forward motion of the anterior end of the ventral cutaneous musculature. There is clearly an antagonistic effect of all *M. costo-cutanei inferiores* and *M. costo-cutanei superiores* which have their cutaneous attachments at the same level of the body, and their phases of activity are likely to be linked by a mechanism of reciprocal excitation-inhibition. This may apply not only to the anterior end of the animal but to the entire length of the body. On the other hand, it is conceivable that the *M. costo-cutanei inferiores* should maintain continuous tension throughout all phases of the locomotory cycle wherever contracting regions of the ventral cutaneous musculature develop sufficient tension to elongate and advance posterior regions. Throughout all phases of elongation, however, tension in the *M. costo-cutanei inferiores* could assist only indirectly the propulsion of the vertebral column by transmitting the tractive force of more anteriorly situated ventral cutaneous musculature.

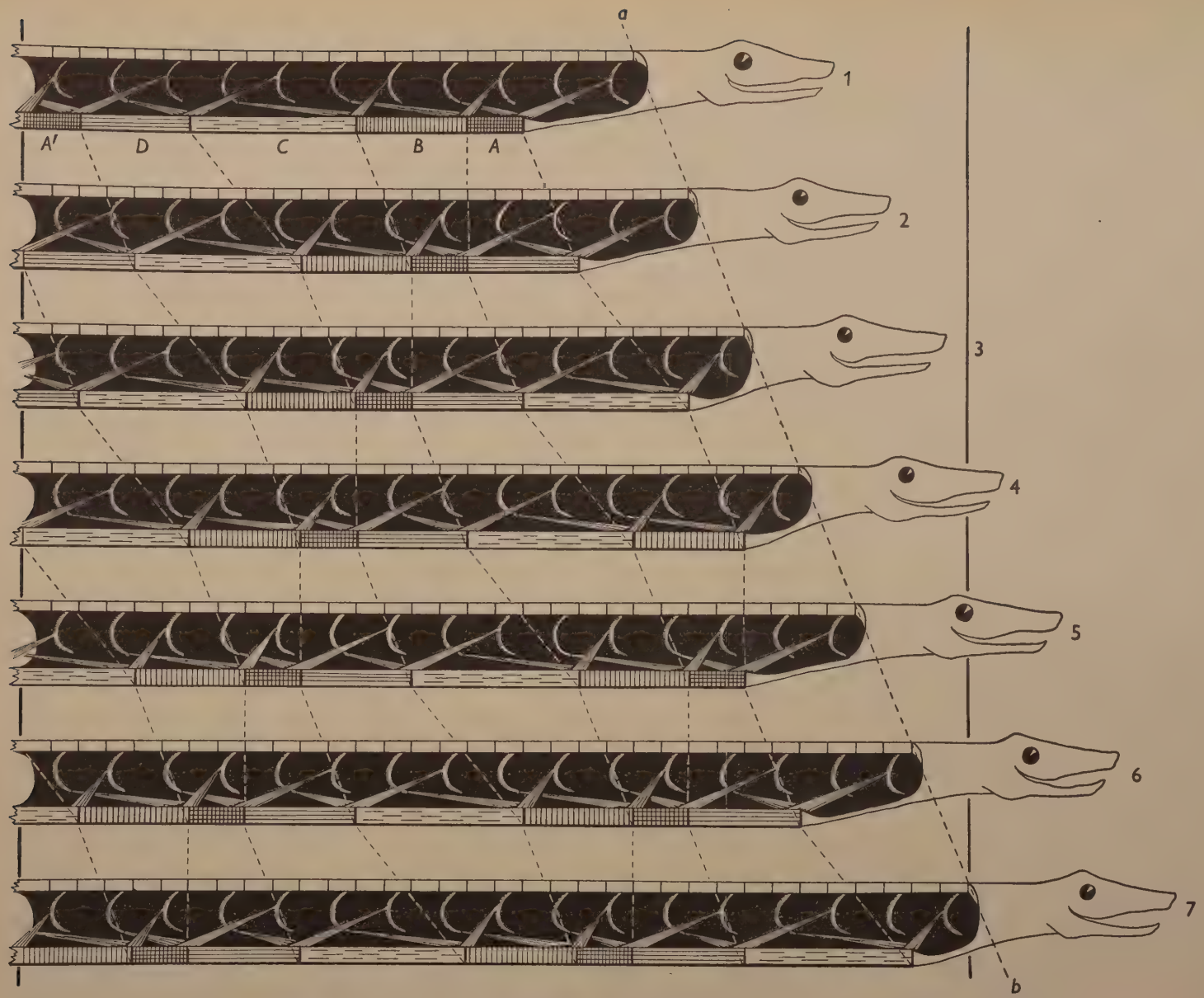

Fig. 7. Diagram illustrating in seven successive positions (1–7) the mechanism of rectilinear progression and the arrangement of muscles involved. On the ventral side the cutaneous musculature is represented in different states of activity. Position 1 shows the anterior region *A* of this musculature in a state of maximal contraction and stationary relative to the ground; region *B* is composed of fibres undergoing contraction; region *C* is in a state of maximal elongation; region *D* is in the process of elongating. In each succeeding position (2–7) these states of activity are shifted in a posterior direction over this muscle, the posterior margin of the maximally contracted region (*A*) remaining fixed to the ground.

The ventral cutaneous musculature and the vertebral column are united by the ribs and two sets of muscles: (i) *M. costo-cutanei inferiores* extending between the tips of the ribs and an anterior region of the ventral skin; these muscles move the vertebral column forward at constant speed (line *ab*); (ii) *M. costo-cutanei superiores* which are attached higher up on the ribs and run to a ventro-lateral region of the skin. These muscles are capable of producing tensions between the vertebral column and the ventral skin and thus to propel the ventral surface forward against frictional resistance. Contractions and extensions of both sets of muscles are related to the state of activity of the ventral cutaneous musculature near which they are attached to the skin. Muscle slips of the two sets with cutaneous attachments at the same level of the body work in strict antagonism; e.g. at the anterior end the *M. costo-cutanei superiores* begin to contract between postions 1 and 2; contraction ceases between 3 and 4. From that moment until midway between postions 5 and 6 these muscles are being passively extended through the forward movement of the vertebral column. In the corresponding anterior *M. costo-cutanei inferiores* contraction and extension are reversed during the same periods.

Although it seems reasonable to suppose that all three groups of muscles contribute propulsive energy to the animal by fulfilling the fundamental requirement of shortening while under tension, it is not easy to demonstrate this conclusively with the present methods. Theoretically, the system could operate without tensions in the ventral cutaneous musculature, if the *M. costo-cutanei inferiores* were able to exert a sufficient force against the posterior edges of the areas of fixation, propelling the vertebral column forward and thus enabling the *M. costo-cutanei superiores* to detach and elongate the anterior margins of the ventrally fixed regions.

In view of the anatomical arrangement, however, it seems more plausible if the vertebral column and all parts rigidly connected with it are considered as a cylinder gliding at constant speed in a tube, or half tube, formed by the skin and the ventral cutaneous musculature which moves forward at varying speeds. The main force required is that for overcoming the external ventral friction and for the successive acceleration of the various regions of the tube. The horizontal, longitudinal arrangement of the ventral cutaneous muscle fibres seems to be best suited for this purpose. This forwardly directed force is balanced by an equal and opposite static reaction under the areas of fixation to the ground. Whatever forward force is imparted to the dorsal cylinder by the *M. costo-cutanei inferiores* must originally be derived from the substrate. The effects of this force are returned to the substrate via the *M. costo-cutanei superiores*.

SUMMARY

1. The rectilinear mode of progression in *Boa occidentalis* is described. It is fundamentally similar to that of an earthworm. The terms 'snail' and 'caterpillar' principles of other authors are inappropriate.

2. During movement, waves of muscular contraction and relaxation pass in a posterior direction over the ventral cutaneous musculature.

3. The ventral surface moves forward in 'steps' while the vertebral column and all parts rigidly connected with it move forward at relatively constant speed. The ribs do not execute any appreciable movement relative to the vertebral column.

4. The probable interaction of the musculature, as deduced from the mechanics and the geometrical arrangements, is discussed.

5. Rectilinear progression is probably a unique type of vertebrate locomotion in so far as it does not involve any lever actions of skeletal structures.

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THE NUTRITIONAL REQUIREMENTS OF *DROSOPHILA MELANOGASTER*

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INTRODUCTION

Drosophila melanogaster lives naturally on various live yeasts that grow in the presence of bacteria, moulds and other micro-organisms, on decaying organic matter. In the laboratory live or autoclaved yeast is perfectly adequate for development, but media on which live yeasts are growing are very heterogeneous; further, the quantity and quality of the available food changes at successive stages in the developing culture. Change in the nature of the substrate probably favours some yeast strains more than others, and thereby alters the food value of the medium (Robertson & Sang, 1944). Frequently such alterations in the medium are unimportant in genetic experiments and may pass unnoticed, although probably much of the environmentally determined variability in exhibition of genes is attributable to this cause. In the case of some genes there is evidence of control of exhibition by specific chemical compounds. Study of such mutants, sensitive to changes in the diet, can be studied best under aseptic conditions, while a completely chemically defined medium might permit detection of these components of the diet which influence exhibition and perhaps throw considerable light on the biochemistry of gene action. Such a medium would also be useful for comparing the effects of different members of allelomorphic series.

Various authors, notably Tatum (1939, 1941) and Gordon & Sang (1941), have attacked aspects of this problem. In particular, Tatum claimed that riboflavine, nicotinic acid and pyridoxin, in addition to an amino-acid source (casein hydrolysate), tryptophane and cholesterol were essential for the development of *Drosophila*, but found it necessary to add a yeast residue consisting of the insoluble fraction remaining after the treatment of autolysed yeast with water and hot alcohol. A somewhat similar 'insoluble' fraction was described by Goldberg, de Meillon & Lavoipierre (1945) for *Aëdes aegypti*. These workers also found that folic acid, and probably biotin, were necessary too.

This paper describes attempts to develop a chemically defined medium, together with methods of sterile culture. A preliminary report has already been published (Begg & Robertson, 1948; Begg, 1949).

EXPERIMENTAL METHODS

(*a*) *General.* Most of the tests have been carried out with an inbred wild type Oregon strain of *Drosophila melanogaster*. Eggs are collected by allowing flies to feed

for 3–4 days on a rich yeast diet, after which they are transferred to bottles fitted with watch-glasses filled with agar (Robertson & Sang, 1944). The eggs are removed by means of platinum spoons fixed into glass holders.

(*b*) *Sterilization.* Great difficulty was encountered in procuring sterile cultures. Many variants have been tried of which the two most successful are as follows:

(1) Eggs are collected and transferred to a test-tube containing sterile water which is rotated about its long axis for several minutes after which the eggs are allowed to settle. The water is then replaced by 50% ethyl alcohol. The tube is again rotated slowly for 3–4 min. and the eggs are allowed to collect at the bottom of the tube, when they are removed with a sterile pipette and transferred to a tube containing 5% antiformin in 10% formaldehyde solution (Glaser, 1943). After 10 min. they are removed to another tube of 50% alcohol, and after settling are transferred in batches of about twenty to small round-bottomed tubes ($\frac{3}{4} \times 2$ in.), containing 50% alcohol. Two further changes are made into similar tubes, so that the total immersion time at this stage is about 25 min. The eggs are then transferred, with the usual precautions, to the sterile culture media. This is best carried out by using pipettes made by drawing out glass tube, squaring the ends off very carefully and plugging with cotton-wool. With the aid of a length of rubber tubing attached to the top of the pipette the operator can suck the eggs into the end of the pipette and gently blow out excess alcohol, while holding the pipette against the glass bottom to prevent the eggs escaping. The entire operation can be carried out by one person after practice, but at first it is safer for two persons to work together.

(2) The treatment here is identical with that already described until the immersion in antiformin and formaldehyde. After this step, eggs are transferred to a short sterile glass tube closed at one end by a platinum grid (100 mesh/in.); they can then be moved from one solution to another in this container. After 10 min. in a mercuric chloride solution,* the eggs are given four 5 min. washes in alcohol. To ensure constant agitation of the sterilizing liquid we have used an electromagnet in conjunction with a small steel washer. Finally, the eggs are removed in batches to the small tubes and thence into the cultures without further transfers.

All cultures are incubated at 25° C. Any culture showing signs of infection is examined by smears and subcultured on to agar slopes of peptone-malt-beef-extract. The commonest infective agency is *B. subtilis*.

EXPERIMENTAL RESULTS

(*a*) *Growth on basal media and yeast autolysates*

Since live or dead yeast is quite adequate for normal development, it was decided to proceed by adding to a basal medium, containing known or likely constituents, extracts of yeast which could be progressively fractionated and replaced by known compounds. To begin with, our primary concern was to obtain basal media which, with the addition of preferably water-soluble yeast extracts, would permit complete

* $HgCl_2$ 0·50 g.
HCl 1·25 ml.
NaCl 6·5 g.
Ethyl alcohol 250 ml.
Water to 1 l.

development. This involved largely empirical modifications of both the basal medium and the yeast extracts; in consequence, many of the early experiments cannot be strictly compared one with another, and are therefore not dealt with. Throughout the work a very large number of yeast autolysate fractions have been tried. Naturally, most of these proved unsatisfactory, and we shall refer only to the more interesting cases.

The primary extract is prepared by autolysing live brewers' yeast for 4 days at 35° C. under reflux with ethyl ether, and filtering and centrifuging the resulting mass until a cell-free solution is obtained. This is then dried under reduced pressure at 40–50° C. Apparently pH conditions alter the composition of the autolysate. Thus when HCl is added to the autolysing mass, little or no growth occurs when larvae are reared on the water-soluble autolysate, while addition of 1% NH_4OH at the start of autolysis yields an extract which is perfectly adequate for normal growth either alone or in conjunction with basal medium. The latter water-soluble extract, prepared in bulk, provided the starting material for the various fractionations, and is referred to throughout as *yeast autolysate*. A highly homogeneous aseptic medium can be prepared by making up an agar gel from this water-soluble extract (0·3 g. autolysate to 5 ml. gel).

When the culture tubes are removed from the autoclave, they are agitated in cold running water to secure even distribution of the components and then sloped or rotated to ensure that a wide area of gel is presented to the larvae; consistency of the gel is an important factor in influencing early growth rate.

The basal medium used in the first experiments included the following constituents: casein, gelatin, aneurin hydrochloride, riboflavine, nicotinic acid, calcium pantothenate, agar, water and salts (or Tatum's salt mixture*). Addition of 0·3 mg. of yeast autolysate was adequate for development within 11 days, compared with 8–9 days on live yeast, and 9–10 days on autoclaved yeast. Reduction of this amount lengthened the larval period. Exhaustive extraction of the dried autolyaste with ether removed no essential constituents.

(*b*) *Fractionation of autolysate*

In the next experiments, we used a fuller basal medium containing, in addition to the components already described, ergosterol, cholesterol, dextrin and pyridoxin hydrochloride. By itself, this medium permitted little larval growth. Addition of biotin, however, did improve growth somewhat, although no pupation enused. When tryptophane and inositol were added, a few small malformed pupae developed after a larval period of 22 days, compared with 6–7 days between egg and pupa on the water-soluble autolysate. The detailed compositions of this and later basal media are quoted in the appendix. This more complete medium (A) was used for experiments on the fractionation of the autolysate. This was started by adding ethyl alcohol to the

* K_2HPO_4 2500 μg. culture; KH_2PO_4 2500 μg. ,,; $MgSO_4$ 1000 μg. ,,; $MnSO_4$ 500 μg. ,,; NaCl 500 μg. culture; $FeSO_4$ 500 μg. ,,; $CaCl_2$ 500 μg. ,,

filtrate from an aqueous solution of the extract to bring the concentration to 50% by volume. The precipitate was filtered off and the filtrate dried under reduced pressure at 40–50° C.

Table 1. *Basal medium A*

Extract: 50 % EtOH-soluble fraction

Quantity (mg.)	Development time (egg to adult) (days)
10	No emergence
50	13
300	11

Table 1 shows that 0·3 g. of this extract is adequate for normal development. Since similar treatment with acetone gave similar results, these are omitted here. Treatment of the 50% alcohol-soluble constituents in aqueous solution with activated charcoal removed essential constituents, both at low and high pH. We were unable to recover these by elution.

Table 2. *Basal medium A*

Extract	Quantity (g.)	Development
80 % alcohol-soluble	0·2	Adults after 22 days
80 % alcohol-insoluble	0·2	Adults after 11 days
90 % alcohol-soluble	0·2	No emergence
90 % alcohol-insoluble	0·2	Adults after 12 days

Higher concentrations of ethyl alcohol left behind essential factors (Table 2). Pupation was found generally to be incomplete when the high alcohol-soluble fractions were supplied; no adults emerged, and larvae usually died in the course of unsuccessful pupation, although larval growth rate was almost normal. Earlier experiments indicated that autolysate could be replaced to some extent by liver extract or liver concentrate.

In view of the work of Goldberg *et al.* (1944) and of our own results, it seemed worth while testing whether failure to pupate might not be due to lack of folic acid. Since folic acid may be removed from solution by precipitation with basic lead acetate (Laland & Klem, 1936), we treated the 50% alcohol-soluble fraction with acetate until no further precipitation occurred. Lead was removed in the usual manner with H_2S; when the gallocyanine test for minute traces of lead proved negative, the solution was dried under reduced pressure and 0·2 g. added to a slightly altered basal medium (B). This medium contained nucleic acid, which was included in view of its high concentration in yeast. The larvae became fully grown at almost the normal rate but failed to pupate. Many crawled round the cultures for days before they finally died. Later, when pure folic acid became available, we found that addition of 1 μg. per culture to the basal medium plus autolysate treated with lead acetate allowed emergence in 11 days. Likewise, addition of folic acid to the 90% alcohol-soluble fraction leads to similar successful pupation (Table 3).

Table 3. *Basal medium B*

Extract	Quantity (g.)	Folic acid (μg.)	Larval growth	Pupation (days)
90 % alcohol-soluble	0·25	—	Normal	—
90 % alcohol-soluble	0·25	1	Normal	7–8
Basic lead acetate filtrate	0·20	—	Normal	—
Basic lead acetate filtrate	0·20	1	Normal	6–7
Basic lead acetate filtrate	0·20	0·2	Normal	9
Basic lead acetate filtrate	0·20	0·04	Normal	13

Further proof of the need for folic acid is shown by the effect of reducing the concentration below 1 μg. per culture. Although the larvae grow quickly, pupation is greatly delayed. It is possible, in view of the low concentration, that the larvae burrow in the medium until they have accumulated a minimal quantity of folic acid, whereafter pupation ensues. From this point onwards, 2 μg. folic acid/culture were added to the basal medium.

Shortly after this, Schultz, St Laurence & Newmeyer (1946) reported the need for choline; this was confirmed, since addition of 1 mg. choline chloride to the basal medium containing folic acid allowed complete though slow development, taking on the average about 15 days. However, it was a considerable advance to have a synthetic medium which would permit complete, although slow, development. Growth-accelerating factor(s) remained to be identified.

(*c*) *Acceleration of larval growth*

Since we possessed a chemically defined medium which would permit complete, though slow development, we now turned to those factors concerned with the acceleration of larval growth. Tatum (1939), Goldberg *et al.* (1944) and Schultz *et al.* (1946) claim that an insoluble yeast fraction is necessary for rapid larval development. This was not consistent with our findings that, in the presence of folic acid, even the 90% alcohol soluble fraction is adequate for rapid development. It seemed likely that differences in the method of preparing the autolysate (unfortunately these authors do not describe their methods of preparation) accounted for the discrepancy, since we have seen already that pH conditions greatly affect the nutritional quality of the preparation. In particular, autolysis in the presence of HCl yields an inadequate extract, while when 1% NH_4OH is added the extract is completely adequate. Apparently some essential component(s) was either left behind or destroyed in the presence of HCl. To test the former possibility, 400 g. live yeast were boiled for $3\frac{1}{2}$ hr. with 500 ml. 0·2 N-HCl. 170 ml. of absolute ethyl alcohol were added to the resulting mass to ease filtration, and the residue was washed several times with 50% alcohol and then dried over a water bath. When 50 mg. were added to basal medium B which contained, in addition, folic acid, growth was completed in 11 days. Of course, such a residue contains the cell-wall constituents of yeast and is an extremely heterogeneous mixture. However, the tests proved the presence of a semi-essential insoluble factor after this treatment. The

factor proved resistant to prolonged oxidation and was not removed by Soxhlet extraction with various organic solvents, including pyridine. Yeast ash did not replace this fraction. Further, when boiled for 1 hr. with concentrations of HCl above 10% inactivation took place. However, when the pyridine extracted HCl residue was boiled with 2% $NaHCO_3$ and filtered, we secured a filtrate which, on neutralization with dilute HCl and addition of alcohol, yielded an active precipitate. When this was added at the rate of 40 mg./culture to the most complete medium development took place in 11 days.

A similarly active precipitate is secured from the treatment of the 2% $NaHCO_3$ extract of whole yeast with HCl and alcohol. Such an extract is best prepared by boiling about 800 g. live brewers' yeast with 1000 ml. 2% $NaHCO_3$ for about 15 min., or until the mass begins to smell distinctly of ammonia. Several filtrations are generally necessary. After treatment of the cell-free filtrate with acid and alcohol, the resulting bulky precipitate is collected on a Buchner filter, washed repeatedly with 50% alcohol, then absolute alcohol and finally ether; it is then dried. The time of boiling apparently influences the potency of the extract.

Thymine requirements

Thymonucleic acid appeared to have a deleterious effect on development. But addition of the base thymine alone in a concentration of 25 μg. per culture accelerated development (Begg, 1949). In the presence of 22 mg. of the $NaHCO_3$ extract development is completed in about 10 days. (See also Ju-Haw Chu, 1945.)

Nature of the $NaHCO_3$ *extract*

The solubility properties of this fraction suggested at first that our medium might be deficient in nucleic acid, although this seemed unlikely from earlier experiments. However, increase in the content of this component leads to no change in development time. The fraction shows a positive HCl-aniline reaction for pentoses and also the Sakaguchi reaction for arginine, suggesting that the protamine adjunct of yeast nucleoprotein might be important, although addition of 20 mg. arginine hydrochloride per culture lead to no improvement. Although these experiments are not far enough advanced to suggest a definite conclusion, it is perhaps significant that addition of globin, prepared from haemoglobin by hydrolysis and salting out, produces pupae of greater size; and that in some nutritional studies it has been suggested that whole casein is sometimes more effective in allowing rapid development, than are its hydrolysis products.

These observations suggest that *Drosophila* may require for rapid growth either some amino-acid supplied in a particular form or possibly a particular short polypeptide molecule. Woolley's (1945) work with strepogenin is suggestive in this connexion. Work is continuing in the hope of identifying this missing component.

DISCUSSION

The best synthetic medium we have to date has the following composition

Casein (vitamin free)	0·06 g.	Aneurin hydrochloride	4 μg.
Gelatin (reprecipitated)	0·045 g.	Riboflavine	6 μg.
DL-Tryptophane	0·005 g.	Nicotinic acid	12 μg.
Ergosterol	0·005 g.	Pyridoxin hydrochloride	12 μg.
Cholesterol	0·005 g.	Biotin	0·2 μg.
Dextrose	0·1 g.	Inositol	1·5 mg.
Agar	0·1 g.	Yeast nucleic acid	3·4 mg.
Water	5 ml.	Calcium pantothenate	12 μg.
Choline chloride	1 mg.	Thymine	25 μg.
Folic acid	2 μg.		

Tatum salt mixture (1936) 6 mg.

It is possible that this does not constitute an optimum relative concentration of components, and some of the vitamins may even be redundant. Further, for completely normal development we still have to add an unknown fraction which is alkali-soluble. Even in the absence of this fraction, however, there are many purposes for which this medium should prove useful. But naturally its use is limited in studies on the relation of gene exhibition to the composition of the diet, since growth still takes a little longer than normally. This may appear unimportant, but since the delay is primarily in the larval stage, we may be dealing with about 25% greater length of larval life, and disregard of this may lead to erroneous conclusions; for the developmental period is recognized as a factor of prime importance in all such work.

SUMMARY

1. Methods are described for the aseptic culture of *Drosophila*.

2. All the factors necessary for normal development can be extracted from yeast in water-soluble form.

3. A chemically defined medium sufficient for almost normal growth is described and includes: casein (and gelatin) as a source of amino-acids, dextrose, cholesterol, ergosterol, yeast nucleic acid, inositol, biotin, aneurin hydrochloride, riboflavine, nicotinic acid, pyridoxin hydrochloride, Ca-pantothenate, choline chloride, thymine and folic acid.

4. For completely normal development an alkali-soluble fraction must be added. This fraction shows nucleoprotein-like reaction, but it is neither arginine nor nucleic acid.

5. Folic acid is essential for pupation.

6. Attention is drawn to the need for caution in gene-action studies with synthetic media which do not give completely normal development even in the wild type.

Part of this work was carried out in 1945, when one of us (M.B.) held a Carnegie Teaching Fellowship. We wish to thank the Trust for their financial help.

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APPENDIX

Basal A

Casein	0·06 g.	Cholesterol	10 mg.
Gelatin	0·045 g.	Aneurin hydrochloride	200 μg.
DL-Tryptophane	0·0012 g.	Nicotinic acid	200 μg.
Dextrose	0·1 g.	Pyridoxin hydrochloride	4 μg.
Agar	0·1 g.	Riboflavine	200 μg.
Salt mixture	0·006 g.	Biotin	4 μg.
Water	5 ml.	Inositol	5 μg.
Ergosterol	10 mg.		

Basal B

Casein	0·06 g.	Tatum salt mixture	0·006 g.
Gelatin	0·045 g.	Aneurin hydrochloride	4 μg.
DL-Tryptophane	0·005 g.	Riboflavine	6 μg.
Ergosterol	0·005 g.	Nicotinic acid	12 μg.
Cholesterol	0·005 g.	Biotin	0·2 μg.
Dextrose	0·1 g.	Pyridoxin hydrochloride	12 μg.
Yeast nucleic acid	0·0034 g.	Calcium pantothenate	12 μg.
Agar	0·1 g.	Inositol	1500 μg.

Water to 5 ml.

Basal C

Casein (vitamin-free)	0·06 g.	Aneurin hydrochloride	4 μg.
Gelatin (reprecipitated)	0·045 g.	Riboflavine	6 μg.
DL-Tryptophane	0·005 g.	Nicotinic acid	12 μg.
Ergosterol	0·005 g.	Pyridoxin hydrochloride	12 μg.
Cholesterol	0·005 g.	Biotin	0·2 μg.
Dextrose	0·1 g.	Inositol	1·5 mg.
Agar	0·1 g.	Yeast nucleic acid	3·4 mg.
Water	5 ml.	Calcium pantothenate	12 μg.
Choline chloride	1 mg.	Thymine	25 μg.
Folic acid	2 μg.		

Tatum salt mixture (1936) 6 mg.

COUNTING SPERMATOZOA

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(With Three Text-figures)

INTRODUCTION

The study of fertilization and spermatozoa often requires a knowledge of the number of spermatozoa in an experiment. Several ways are available of counting spermatozoa or of assigning a number, related to sperm density, to a suspension. The most obvious of these are:

(1) *Haemocytometer counts.* This is a tedious and time-consuming method. The delay involved in making a count precludes the possibility of making up suspensions of known concentration before an experiment, unless another and quicker method of estimating sperm density is used as well.

(2) *Estimation of total nitrogen.* This method, used by Gray (1928), has some of the disadvantages of (1) and is not accurate, because the seminal plasma of sea-urchin semen contains protein which contributes to the total nitrogen measured. At the same time the proportions of spermatozoa and seminal plasma vary in different samples of semen. Information on this latter point is given later.

(3) *Weighing dried semen.* This method is not suitable for rapid standardization. Apart from the question of its accuracy, it suffers from some of the defects referred to in (2).

(4) *Visual comparison with standard opacity tubes.* This method is used in field experiments involving mammalian spermatozoa (Salisbury, Beck, Elliott & Willett, 1943). It is less suitable for sea-urchin spermatozoa where different species may have different light-absorbing and light-scattering properties.

(5) *Electrical resistance of sperm suspension.* So far as is known, the possibility that the electrical resistance of a suspension may vary with the concentration of spermatozoa has not been examined. Even if a relationship does exist between sperm density and resistance, the method requires special apparatus in the form of an alternating-current bridge and electrodes. If, in the future, the electrical method of measuring mammalian sperm activity (Rothschild, 1948) is found to be of value, the possibility of counting spermatozoa by this means might be explored, as the same apparatus would be used both for measuring sperm activity and counting them.

(6) *Absorptiometric measurement of opacity.* The application of this method to suspensions of sea-urchin spermatozoa is the main subject of this paper. Salisbury *et al.* (1943) compared the haemocytometric and nephelometric methods of estimating bull-sperm density. When comparing the two methods, it must be remembered that semen consists of spermatozoa and seminal plasma, both of which may absorb light and one of which, spermatozoa, has light-scattering qualities. There may therefore be a difference between washed and unwashed spermatozoa. Many studies of

mammalian spermatozoa are carried out on washed spermatozoa, i.e. spermatozoa which have been centrifuged and re-suspended in some form of Ringer solution, the supernatant seminal plasma having been removed. Some investigations have been carried out on washed sea-urchin spermatozoa (Barron, Seegmiller, Mendes & Narahara, 1948), but in view of the difficulties encountered in re-suspending centrifuged sea-urchin spermatozoa satisfactorily, it would be unwise to assume that the concentrations of sperm in samples examined before and after centrifugation followed by 're-suspension' to the same volume, are necessarily the same. Furthermore, it must be shown that a solution such as sea water or Ringer phosphate has similar light-absorbing properties to seminal plasma, or that the effect of spermatozoa in preventing light impinging on the photocell is large compared with the effect of seminal plasma.

MATERIAL

Unwashed and washed spermatozoa of *Echinus esculentus*.

METHOD

Haemocytometer counts. Fuchs-Rosenthal haemocytometer; depth of chamber, 200 μ. The semen or sperm suspension was diluted 25,000 times, formalin being added to kill the spermatozoa. Sperm counts on four randomly selected millimetre squares (each divided into sixteen subsquares), were made in each experiment. The total number of spermatozoa counted was 600–1000.

Photoelectric absorptiometer. Spekker (A. Hilger, Ltd.) with filters H503 and H608. The former is a 'Calorex' heat filter, the latter Ilford's 'Spectrum Red'. The cells were 1 cm. long.

Centrifugation. Semen was centrifuged at 8000 r.p.m. in an angle centrifuge for 10 min. The procedure was repeated three times, the sperm being re-suspended each time in sea water.

RESULTS AND ANALYSIS

Comparison of haemocytometer and absorptiometer estimates. A typical experiment is shown in Table 1. In this table, the letters *A2*, *C4*, *B6* and *D8* refer to particular square millimetres in the haemocytometer.

The experiment was repeated ten times, giving fifty comparisons of sperm concentration in the absorptiometer cell and absorptiometer reading. The scatter diagram for these comparisons is shown in Fig. 1.

Denoting these fifty co-ordinates by x_1y_1–$x_{50}y_{50}$, where x is the concentration of spermatozoa in the absorptiometer and y the absorptiometer reading, the logarithmically linear curve which best fits the co-ordinates x_1y_1–$x_{50}y_{50}$, in the sense of least squares, when the error in x is great compared with that in y, is

$$y = 2e^{-x/p}, \quad p > 0 \tag{1}$$

where

$$p = -\log_{10}e \frac{\sum_{i=1}^{50} \{x_i(\log_{10}y_i - \log_{10}2)\}}{\sum_{i=1}^{50} \{\log_{10}y_i - \log_{10}2\}^2}. \tag{1·1}$$

This curve is shown in Fig. 2.

Table 1

Sperm counts									
A2	12	10	6	9	*C4*	8	6	9	7
	8	14	8	18		17	9	15	7
	16	16	14	12		10	12	18	12
	18	16	10	13		11	13	14	8
B6	7	11	7	8	*D8*	10	13	17	7
	6	18	9	8		7	14	14	11
	16	7	11	8		12	14	11	13
	9	9	16	10		8	10	10	9

Average number of sperm per 0·2 mm.^{3}, 179
Estimate of original sperm density, $179 \times 1{\cdot}25 \times 10^8 = 2{\cdot}238 \times 10^{10}$
Standard error of estimate, $\sqrt{(716)} \times 0{\cdot}313 \times 10^8 = 8{\cdot}362 \times 10^8 = 3{\cdot}7\,\%$

Absorptiometer measurements		
Subsample no.	No. of sperm/ml., after dilution, calculated from haemocytometer count	Absorptiometer reading
1	$3{\cdot}197 \times 10^8$	0·432
2	$2{\cdot}558 \times 10^8$	0·533
3	$1{\cdot}918 \times 10^8$	0·694
4	$1{\cdot}279 \times 10^8$	0·924
5	$0{\cdot}639 \times 10^8$	1·355
6	0 (sea water blank)	2·000

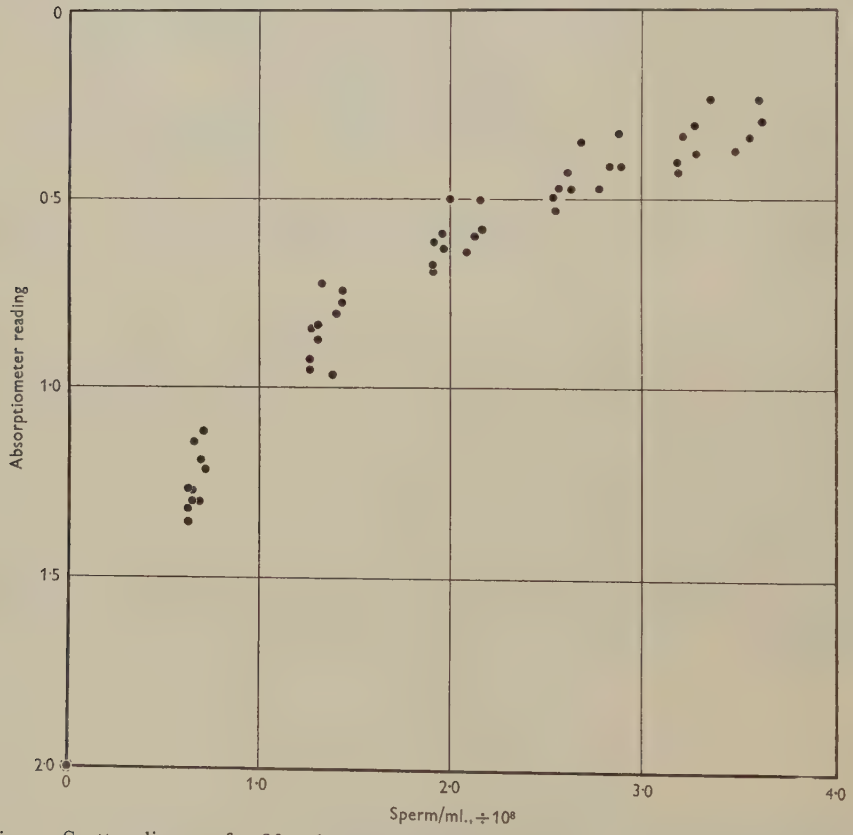

Fig. 1. Scatter diagram for fifty absorptiometer reading-sperm concentration comparisons, *Echinus esculentus*.

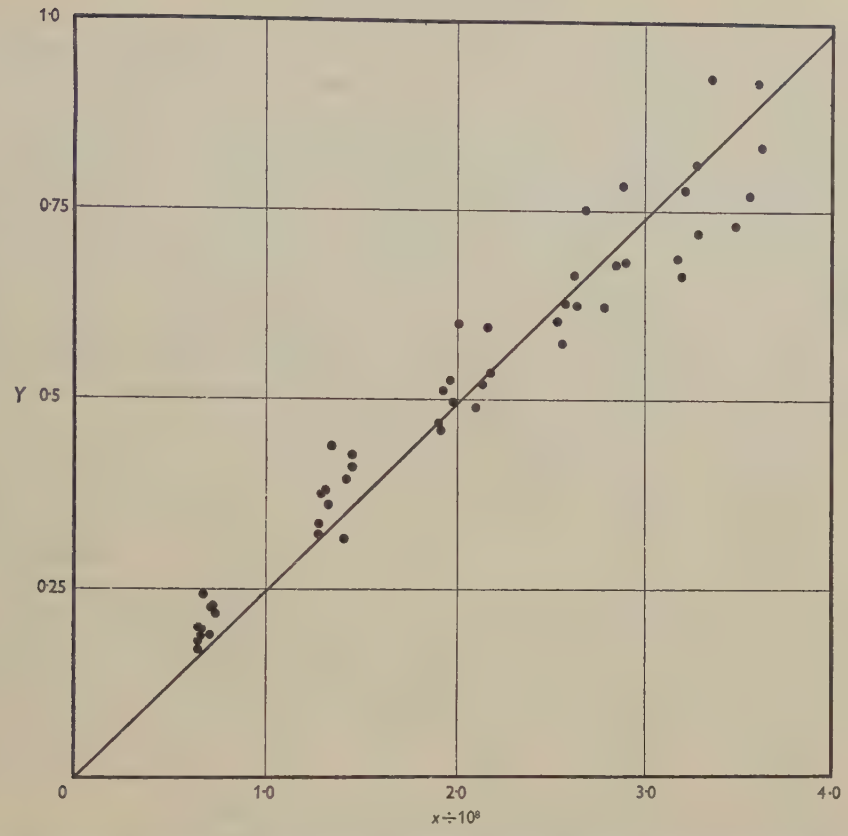

Fig. 2. Graph of $y=2e^{-x/p}$. Ordinates, $Y=\log_{10}2-\log_{10}y$, where $y=$absorptiometer reading; abscissae, x, the number of sperm/ml. in the absorptiometer; p is calculated from equ. (1·1). The individual points were obtained from the same data as those in Fig. 1, with co-ordinates Y, x instead of y, x.

Suppose that an unknown sample of semen contains N sperm/ml., and that it is diluted d times, giving an absorptiometer reading y'; p is known from equ. (1·1) and x', the concentration of spermatozoa in the absorptiometer, can therefore be calculated, using equ. (1) in the form

$$x'=2{\cdot}3026\,p(0{\cdot}3010-\log_{10}y'). \qquad (1{\cdot}2)$$

The estimate of the original concentration of spermatozoa in the sample is dx'/ml. The standard error of x' is

$$\sqrt{\frac{125\times10^6}{4}}\times\sqrt{\frac{x'}{d}} \quad \text{and} \quad N=dx'\pm\left\{\sqrt{\frac{125\times10^6}{4}}\times\sqrt{(dx')}\right\}$$
$$=dx'\pm5590\sqrt{(dx')}. \qquad (2)$$

This result can be expressed more accurately as follows:

$dx'-1{\cdot}645\,(5590)\sqrt{(dx')}\leqslant N\leqslant dx'+1{\cdot}645\,(5590)\sqrt{(dx')}$, confidence coefficient, 0·90;

$dx'-1{\cdot}960\,(5590)\sqrt{(dx')}\leqslant N\leqslant dx'+1{\cdot}960\,(5590)\sqrt{(dx')}$, confidence coefficient, 0·95;

$dx'-2{\cdot}576\,(5590)\sqrt{(dx')}\leqslant N\leqslant dx'+2{\cdot}576\,(5590)\sqrt{(dx')}$, confidence coefficient, 0·99.

A rough test of the accuracy of the absorptiometric method of counting spermatozoa can be made by calculating the original sperm concentration from the five absorptiometer readings in Table 1. Bearing in mind that the original semen contained $2{\cdot}238 \times 10^{10}$ sperm/ml. and was diluted 70, 87·5, 116·7, 175, and 350 times for subsamples 1–5 respectively, the following estimates are obtained for the original sperm concentration: subsample 1, $1{\cdot}885 \times 10^{10}$; subsample 2, $2{\cdot}033 \times 10^{10}$; subsample 3, $2{\cdot}170 \times 10^{10}$; subsample 4, $2{\cdot}374 \times 10^{10}$; subsample 5, $2{\cdot}395 \times 10^{10}$. The figures differ from the original haemocytometer estimate by the following percentages: −16, −9, −3, +6 and +7. Except in the case of subsample 1, the results are in reasonable agreement. Fig. 2 clearly shows the scatter in the low absorptiometer reading range; in practice, the original suspension is diluted sufficiently, that is 200 times, for the reading to fall on the lower half of this curve.

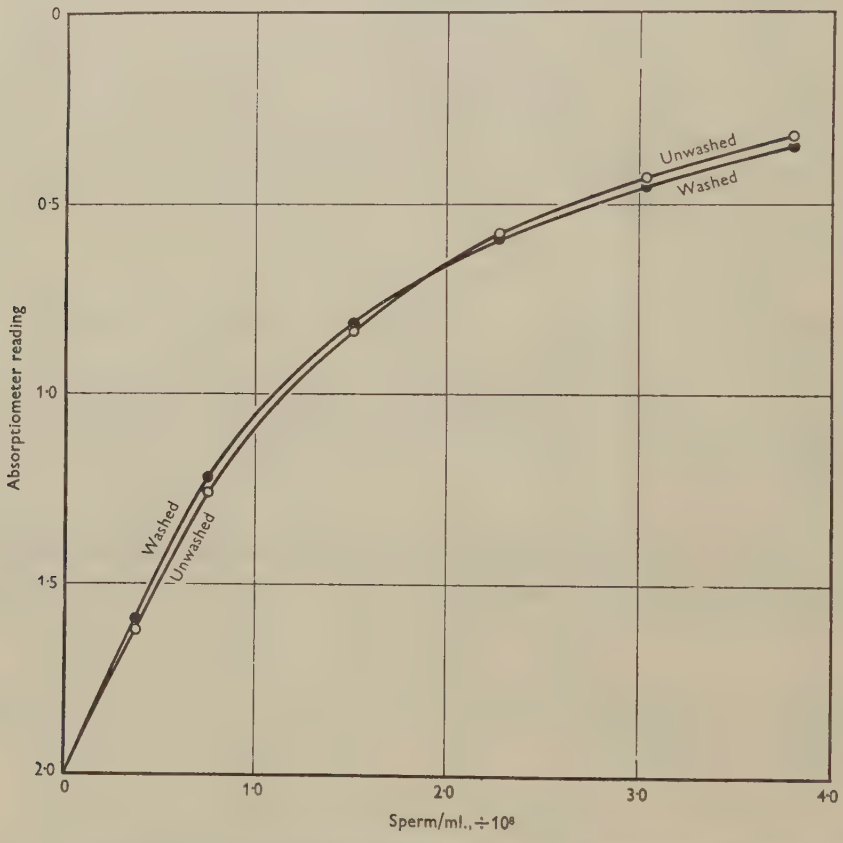

Fig. 3. Comparison of optical properties of washed and unwashed spermatozoa of *Echinus esculentus*.

There is internal evidence from actual readings that the accuracy of the absorptiometric method of counting spermatozoa is equivalent to a haemocytometer count of about 950 spermatozoa (see 'Accuracy of the Absorptiometer'

later). As the standard error of an estimate based on counting 950 spermatozoa is approximately $d\sqrt{(950)}$ ('Student', 1907), the accuracy of the absorptiometer is about $\pm 3\%$.

Comparison of light-absorbing and light-scattering properties of washed and unwashed semen. Fig. 3 is a comparison of the absorptiometer reading-sperm concentration plot for semen diluted in the usual way (unwashed sperm) and washed spermatozoa. The latter were obtained by centrifuging the spermatozoa, pipetting off the seminal plasma and re-suspending the sperm in sea water, three times. The curves are identical, within the limits of experimental error. This is due to the effect of light-scattering and light absorption by the spermatozoa predominating over the effect of light-absorption by seminal plasma.

Variations in number of sperm/ml. in semen. Reference was made in the Introduction to variations in the number of sperm/ml. in samples of semen from different urchins. In ten samples selected at random the number of sperm/ml. $\div 10^{10}$ were 1·81, 2·48, 2·58, 1·97, 2·89, 2·73, 2·23, 2·24, 2·30, 2·53. The maximum difference in these samples is 60%. The density of semen was found to be about four times that of seminal plasma.

Weight of semen. Measurement of the dry weight of semen is markedly inferior to absorptiometer measurements as a method of estimating the number of sperm in a sample. In view of the time taken to complete such measurements and the difficulty of disentangling the influence of sperm and seminal plasma, with their different densities, on the dry weight, this method of counting spermatozoa cannot be recommended.

ACCURACY OF THE ABSORPTIOMETER

In a single set of five readings, with different dilutions of the same suspension, the ratio of the concentrations will not be affected by errors in the haemocytometer count, assuming that dilution is carried out accurately. Errors due to this cause and to absorptiometer reading errors, including zero errors or non-linearity of the logarithmic readings, can be estimated by making comparisons within sets of five readings. If x is the true concentration and Y the logarithmic departure of the absorptiometer readings from 2, the five readings will give an estimate of the calibration constant

$$b = \frac{\Sigma(xY)}{\Sigma(Y^2)}, \tag{3}$$

and this will minimize the sum of squares $\Sigma(x - bY)^2$, which on substitution of equ. (3) becomes

$$\Sigma(x^2) - \frac{\{\Sigma xY\}^2}{\Sigma(Y^2)}. \tag{4}$$

For forty degrees of freedom within the ten sets of five readings, the minimized sum of squares is 1·0786, corresponding with a mean square 0·02697. The average value of b for the series is 1·7805 and the average value of $\Sigma(Y^2)$ is 8·0849. The sampling variance of b is

$$V(b) = \frac{V'(x)}{\Sigma(Y^2)}, \tag{5}$$

where V' is the mean square residual.

For true precision we require $b^{-2}V(b)$ which approximately equals $(1{\cdot}78)^{-2} \times 0{\cdot}02697 \times (8{\cdot}0849)^{-1} = (950)^{-1}$, showing that the precision of b gives an estimate of x equivalent to making a count of about 950 spermatozoa. This applies to dilutions giving high absorptiometer readings, which as mentioned earlier on, is done in practice.

Bull semen

Because of its practical importance, a similar analysis of nine samples of bull semen was carried out. The value of p (see equ. (1·1)) was found to be $4{\cdot}8520 \times 10^7$. The number of spermatozoa N per ml. of semen is given by the equation

$$N = (3{\cdot}3515 \times 10^9)\,(0{\cdot}3010 - \log_{10} a), \tag{6}$$

where a = absorptiometer reading.

It is important to note that this equation only applies when the semen is diluted 30 times before being put into the absorptiometer, and when the absorptiometer is set to 2 for the blank with phosphate buffer, etc., in the cell. If another blank setting is used, for example m, $\log_{10} m$ must be substituted for $\log_{10} 2$ in equs. (1·1), (1·2) and (6). The accuracy of N in equ. (6) is about $\pm 1{\cdot}6\%$. Equ. (6) was obtained from data relating to three bulls. If nine different bulls had been used, the estimate would probably have been somewhat less accurate. The experimental procedure may be summarized as follows:

(1) Set Spekker drum to 2·000 with Ringer phosphate in cell.

(2) Dilute bull semen 30 times and put in cell in Spekker.

(3) Obtain Spekker reading a, for example 0·90:

$$\log_{10} a = \bar{1}{\cdot}9542 = -0{\cdot}0458. \quad \{0{\cdot}3010 - \log_{10} a\} = 0{\cdot}3468.$$

(4) No. of sperm/ml. semen, $3{\cdot}3515 \times 10^9 \times 0{\cdot}3468 = 1{\cdot}16 \times 10^9$.

SUMMARY

1. The number of spermatozoa in a sample of sea-urchin semen (*Echinus esculentus*) can be accurately estimated by measuring the amount of light scattered and absorbed by a subsample composed of semen diluted with sea water. A 'Spekker' photoelectric absorptiometer was used for the measurements, with a cell 1 cm. long.

2. The number N of spermatozoa in a sample of sea-urchin semen was found to be given by the equation

$$N = 8{\cdot}0918 \times 10^{10} Y' \pm (1{\cdot}590 \times 10^9 \sqrt{Y'})$$

where $Y' = 0{\cdot}3010 - \log_{10} y'$ and y' = absorptiometer reading, when the original sample was diluted 200 times and the absorptiometer set to 2·000 for the blank.

3. The light-scattering and light-absorbing properties of washed and unwashed suspensions were found to be virtually identical.

4. The number of spermatozoa in a sample of bull semen was found to be given by the equation $N = 3{\cdot}3515 \times 10^9 Y' \pm (5{\cdot}283 \times 10^7 \sqrt{Y'})$, when the original sample was diluted 30 times and the absorptiometer set to 2·000 for the blank.

I am much obliged to Prof. R. A. Fisher, F.R.S., for his advice on the statistical parts of this paper, and in particular on the section entitled 'Accuracy of the Absorptiometer'.

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THE PHYSIOLOGY OF SEA-URCHIN SPERMATOZOA

CATALASE

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(With Five Text-figures)

INTRODUCTION

The existence of catalase in the semen of *Echinus esculentus* was briefly mentioned in a previous paper (Rothschild, 1948*a*) and a similar observation was made by Evans (1947) using the sperm or semen of *Arbacia punctulata*. Quantitative determinations of catalase content were not made in either case; nor was the presence of catalase in spermatozoa, as opposed to seminal plasma, established. Mammalian spermatozoa contain negligible quantities of catalase, its presence even being used on occasions as an index of bacterial contamination (Blom & Christensen, 1947). The existence of this enzyme in significant quantities in sea-urchin semen is therefore of special interest, though it must be remembered that sea-urchin and mammalian spermatozoa have somewhat different types of metabolism. The former are not motile in the absence of O_2 (Harvey, 1930) and exhibit negligible aerobic or anaerobic glycolysis (Rothschild, 1948*b*). The latter produce considerable quantities of lactic acid, 2 mg./10^9 sperm/hr. at 37° C. anaerobically, and 50–90% of this figure aerobically (Mann, 1948), while movement is not dependent on the presence of O_2.

The main function of catalase is believed to be the decomposition of H_2O_2, formed during metabolism. Keilin & Hartree (1936) have suggested that catalase may have a peroxidatic function as well, in the sense of catalysing coupled oxidations of the type $C_2H_5OH + H_2O_2 \rightarrow CH_3CHO + 2H_2O$. Zeller & Maritz (1944) have described another role of catalase, in the interaction between L-ophioamino acid oxidase and its substrates, which follows different pathways in the presence and absence of catalase. In this paper, the expression 'catalatic activity' refers to the catalytic decomposition of H_2O_2 by catalase into O_2 and H_2O.

Evans (1947) refers to the toxic action of H_2O_2 on sea-urchin spermatozoa. His results can only be interpreted as showing that these spermatozoa are unable to decompose H_2O_2 added directly or indirectly to the external medium. This may be thought surprising as sea-urchin semen contains catalase, though H_2O_2 is also very toxic to a number of catalase-containing bacteria (Lwoff & Morel, 1942).

In order to clarify the position it is first necessary to make quantitative measurements of the catalatic activity of sea-urchin semen. Secondly, an answer must if possible be found to the question as to whether the catalase is in the spermatozoa or in the seminal plasma. This may involve a knowledge of the relative amounts of sperm and seminal plasma in a sample of semen; the actual number of spermatozoa per ml.

of semen does not by itself provide this information unless the volume of an individual spermatozoon is also known. The volume ratio obtained by centrifugation is rather inaccurate even at high centrifugal speeds (Shapiro, 1935), something which is likely to be aggravated by the long tail of the sea-urchin sperm and by the peculiar shape of the head. Catalatic activity is defined in terms of the dry weight of the material being examined. The dry weight of a unit quantity of seminal plasma, separated from semen by centrifugation, can readily be obtained. But even if the spermatozoa can be completely freed of seminal plasma by repeated washing, the dry weight of the centrifuged spermatozoa will include solids derived from the liquid used in the washing process, it being of course impracticable to use distilled water. Moreover, the process of washing sea-urchin spermatozoa may leach substances, possibly even catalase, out of or off the surfaces of the spermatozoa. This was mentioned in previous papers (Rothschild, 1948*a*, *b*) in which it was shown that the supernatant fluid obtained by centrifuging suspensions of spermatozoa diluted with sea water and allowed to metabolize for some hours maintained the respiration of other spermatozoa suspended in it at a higher level than controls in ordinary sea water. Furthermore, during centrifugation, Androgamone I diffused out of the spermatozoa into the seminal plasma.

The problems outlined in the previous paragraph are the subject of this paper.

MATERIAL

Semen, spermatozoa and seminal plasma of *Echinus esculentus*.

EXPERIMENTAL PROCEDURE

Measurement of catalatic activity. Measurements were made in differential manometers, $k_{O_2} \doteqdot 1{\cdot}7$. The vessels were conical, with a capacity of about 17 ml., and had no centre cups or side-arms. The biological material was suspended in neutral phosphate buffer, sea water, or in some cases specifically mentioned, distilled water, 4 ml. of the suspension being placed in the manometer vessel. 0·1 ml. of H_2O_2 solution of known concentration was added to the suspension at $t=0$. Readings were taken at 1 min. intervals for 5 min., a later reading being made to determine the total O_2 evolved. The H_2O_2 solution was added from a glass dangling cup, capacity 0·2 ml., suspended in the main vessel and released magnetically at $t=0$. In a previous paper (Laser & Rothschild, 1947) an electromagnetic mixer, suitable for such purposes, but intended for measurements of O_2 uptake immediately after fertilization, was described. This device has certain drawbacks, the more important of which are: (1) the capillary tubing of the manometer has to be broken for incorporation of the mixer, and subsequently rejoined; (2) unless special precautions are taken there is a danger of asymmetrical heating effects due to the flow of current through the solenoids which produce the magnetic field; (3) unless elaborate safety devices are incorporated, there is a possibility of time-consuming accidents due to supply failures if the mixer is mains-operated. The new method of mixing H_2O_2 with the catalase-containing suspension (Fig. 1) is less complicated, cheaper, and generally superior. The glass dangling cup *D* has fused in it a piece of fine platinum

wire terminating in a hook, *H'*. When the cup has been filled with the H_2O_2 solution it is hooked on to the platinum wire *W*, which also terminates in a hook *H*. The platinum wire passes up the bore of the manometer capillary, and at its distal end is joined to a piece of iron wire, *I*. The magnet *M* holds the iron wire, and therefore

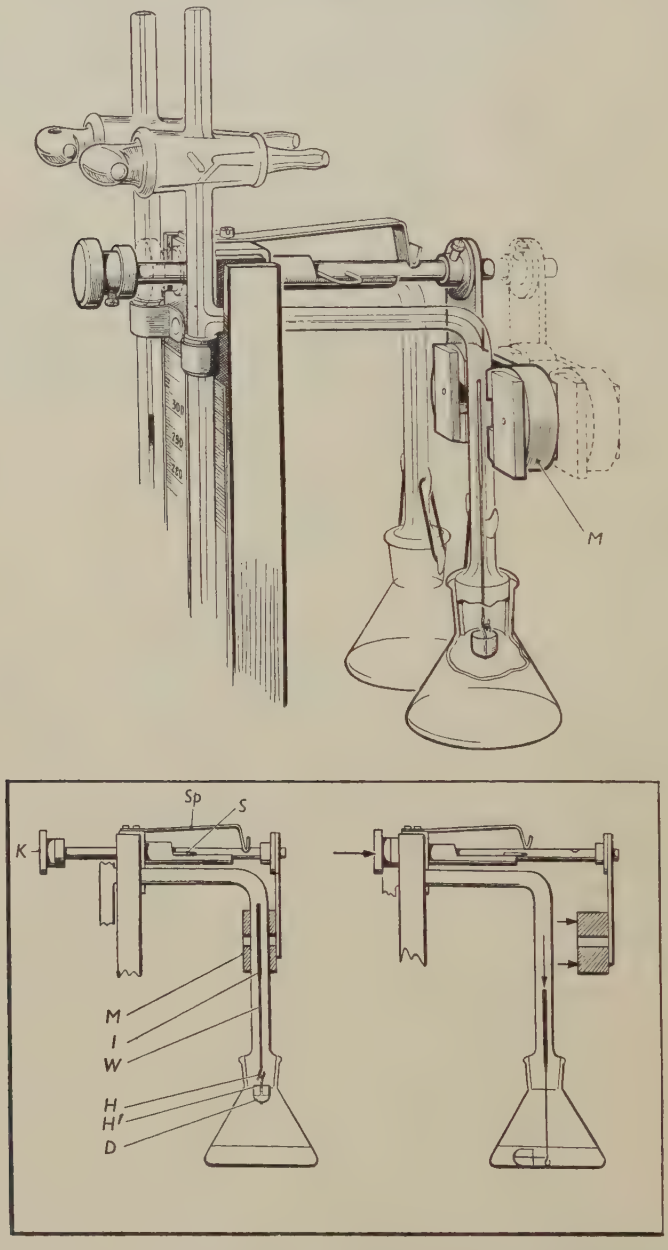

Fig. 1. Magnetic mixer. *D*, dangling cup containing H_2O_2 solution; *H'*, platinum hook; *W*, platinum wire terminating in platinum hook *H*; *I*, iron wire; *M*, magnet; *K*, release knob; *Sp*, locating spring, *S*, anti-rotation stop.

the platinum wire and the dangling cup, above the suspension while the manometer is being shaken, in these experiments at 170 c.p.m. with a 3·5 cm. stroke. Surface tension prevents the H_2O_2 solution splashing out of the cup even at such high shaking speeds. At $t=0$ the release knob K is pushed in and the cup falls into the suspension in the main vessel. In practice, the shaker speed is kept at 95 c.p.m. during the 10 min. equilibration time, and as t approaches 0 it is gradually increased to 170 c.p.m., the index finger of the right hand being kept on the release knob. With a dangling cup of 0·2 ml. capacity containing 0·1 ml. of H_2O_2 solution, 4 ml. of fluid in the main vessel was found to be the optimal amount for rapid mixing and diffusion, one of the limiting factors in this method of measuring catalatic activity. Perspex dangling cups, though easy to make, were found to be less satisfactory than glass ones as they tend not to sink at once after being dropped; also, after release, they cannot be heard rattling in the main vessel.

All experiments were done at 15° C. with air in the gas phase of the manometers.

The H_2O_2 solution in the dangling cups was equivalent to about 1500 μl. O_2/ml. and was prepared by diluting 95 vol. H_2O_2 with glass-distilled H_2O. The concentration of the H_2O_2 solution was checked periodically by titration with $KMnO_4$ in the presence of H_2SO_4 and also by measuring the amount of O_2 liberated from 0·1 ml. H_2O_2 solution when this was added to horse-liver catalase in neutral phosphate buffer in the main vessel.

There are various methods of expressing catalatic activity, most of which have their own advantages and disadvantages. In this paper it is defined by the equation

$$A_c = \frac{1}{gt} \ln \frac{a}{a-x},$$

where g = dry weight in g./ml. of the catalase-containing material used in the actual experiment; t = time, in all experiments, 1 min.; a = initial substrate concentration, i.e. H_2O_2 concentration expressed in μl. O_2; x = amount of H_2O_2, expressed in μl. O_2, decomposed in 1 min.

For purposes of comparison, a protocol is given in an Appendix showing the catalatic activity of the writer's blood. This, and the details given in the Results section, enable A_c to be compared with 'Kat. F' (Sumner & Somers, 1943), 'Q-Kat.', (v. Euler, 1934), or any other method of expressing catalatic activity such as the velocity constant k_1 of Bonnichsen, Chance & Theorell (1947), to which A_c is closely related. The catalatic activity of different solutions cannot be compared purely on the basis of the dilution necessary to produce, for example, 70 μl. O_2 in the first minute, from an H_2O_2 solution containing 150 μl. O_2, as the solutions may not be comparable in regard to their content of solid material. In comparing the catalatic activity of blood and semen, therefore, attention must not only be paid to the dilution but also to the dry weight of a unit volume of the suspension used in an actual experiment.

The range over which there is a linear relationship between enzyme concentration and rate of O_2 evolution is limited. The reason for this at low enzyme concentrations is the destruction of catalase by H_2O_2; when the enzyme concentration is too high, the rate of O_2 evolution is affected by diffusion limitations and by the inertia of the

measuring system. The amounts of semen and seminal plasma were adjusted as far as was possible so that O_2 evolution was in the linear range.

Respiration of spermatozoa. The O_2 uptake of spermatozoa was examined in the presence of H_2O_2, neutralized hydroxylamine hydrochloride ('hydroxylamine') and H_2O_2 + hydroxylamine. The experiments were carried out in differential manometers $k_{O_2} \doteqdot 1{\cdot}2$, reagents being added from side-arms. The main vessels contained 2·0 ml. sperm suspension and the centre cups 0·2 ml. 10% KOH and filter-papers. The temperature was 15° C., the gas phase was air, and the shaking rate was 100 c.p.m. with a 3·5 cm. stroke.

Sperm counts. Photoelectric absorptiometer (Rothschild, 1950).

RESULTS

Catalatic activity of semen. The catalatic activity of semen in sea water was found to be 50 (average of 16 determinations) and of seminal plasma 210 (average of 8 determinations). From these figures the catalase content of seminal plasma appears to be four times greater than that of semen; this makes it difficult, if not impossible, to correlate catalatic activity with seminal sperm density, as the catalatic activity of the spermatozoa appears to be constantly overshadowed by that of the medium.

Estimations of catalase content involve measurements of the *rate* of evolution of O_2 from H_2O_2. There is no doubt that sea water is an unsatisfactory medium in which to make such measurements on spermatozoa. Sea water, being the normal environment of ejaculated spermatozoa, is not harmful to them; consequently there is a delay before the added H_2O_2 reacts with the catalase in the spermatozoa, making the activity of semen appear lower than it really is. This difficulty does not apply in the case of seminal plasma in which there are unlikely to be any surfaces which might impede the interaction between catalase and H_2O_2. The catalatic activity of semen in neutral phosphate buffer, which is toxic to spermatozoa, was found to be twice that in sea water (Table 1).

Table 1. *Catalatic activity of semen*, Echinus esculentus

	V_1	V_2	V_3	V_4
Main vessel	4 ml. S Cup + 0·1 ml. P Wire	3 ml. S 1 ml. B Cup + 0·1 ml. P Wire	2 ml. S 3 ml. B Cup + 0·1 ml. P	1 ml. S 3 ml. B Cup + 0·1 ml. P
μl. O_2 evolved in 1 min.	103	91	66	34
Dry weight of 1 ml. semen, 150 mg. No. of sperm/ml. semen, $2{\cdot}81 \times 10^{10}$. S, 1 ml. semen + 10 ml. 0·025 M neutral phosphate buffer. B, 0·025 M neutral phosphate buffer. P, H_2O_2 solution containing 1490 μl. O_2/ml.				
A_6	90	90	90	80

The difference between the catalatic activity of semen in sea water and phosphate buffer is strong evidence for the existence of catalase in spermatozoa apart from its

presence in seminal plasma, though phosphate buffer may increase the activity of the catalase which is present. An experiment to demonstrate this point, but comparing the catalatic activity of semen in sea water and distilled water, which is of course as toxic as phosphate buffer, is given in Table 2. The higher activity of semen in distilled water strongly suggests that catalase diffuses out of the spermatozoa (or H_2O_2 in) more readily when they are in this medium than when in sea water.

Table 2. *Catalatic activity of semen*, Echinus esculentus

	V_1	V_2
Main vessel	3·65 ml. sea water 0·35 ml. semen Cup + 0·1 ml. P Wire	3·70 ml. dist. H_2O 0·30 ml. semen Cup + 0·1 ml. P Wire
μl. O_2 evolved in 1 min.	56	101
Dry weight of 1 ml. semen, 157 mg. P, H_2O_2 solution containing 1550 μl. O_2/ml. No. of sperm/ml. semen, $2{\cdot}79 \times 10^{10}$.		
A_c	30	90

Effect of washing on catalatic activity of spermatozoa. Confirmation of the existence of catalase in spermatozoa is obtained from experiments in which semen is washed with sea water (Table 3). If the apparent catalatic activity of semen were only due to seminal plasma, spermatozoa which have been once washed with sea water should have a lower catalatic activity than sperm in seminal plasma. Table 3 shows that this is not the case. The same result is obtained when the semen is repeatedly washed, by centrifugation, removal of supernatant fluid, and re-suspension to the original sperm

Table 3. *Catalatic activity of semen, seminal plasma and washed spermatozoa*, Echinus esculentus

	V_1	V_2	V_3	V_4
Main Vessel	3·7 ml. phosphate buffer 0·30 ml. semen Cup + 0·1 P Wire	3·7 ml. phosphate buffer 0·30 ml. 1st plasma* Cup + 0·1 P Wire	3·7 ml. phosphate buffer 0·30 ml. 2nd plasma* Cup + 0·1 P Wire	3·7 ml. phosphate buffer 0·30 ml. washed spermatozoa Cup + 0·1 P Wire
μl. O_2 evolved in 1 min.	92	52	48	89
Dry weight of 1 ml. semen, 150 mg. Dry weight of 1 ml. seminal plasma, 40 mg. Dry weight of 1 ml. washed spermatozoa, 125 mg. P, H_2O_2 solution containing 1550 μl. O_2/ml. No. of sperm/ml. semen in V_1 and V_4, $2{\cdot}85 \times 10^{10}$.				
A_c	80	140	130	90

* 1st plasma, after 20 min. centrifugation at 1100 g.; 2nd plasma, after a further 20 min. centrifugation at 1100 g., without re-suspension.

density by addition of sea water. This procedure reduces the catalatic activity of the seminal plasma because of its dilution with sea water, but the catalase content of the final suspension, which contains sperm in sea water, is not correspondingly reduced. In some experiments, particularly with the semen of *Psammechinus miliaris* which were done the previous year, the reduction in catalatic activity of seminal plasma was not proportional to its dilution; moreover, in some cases there was an increase in

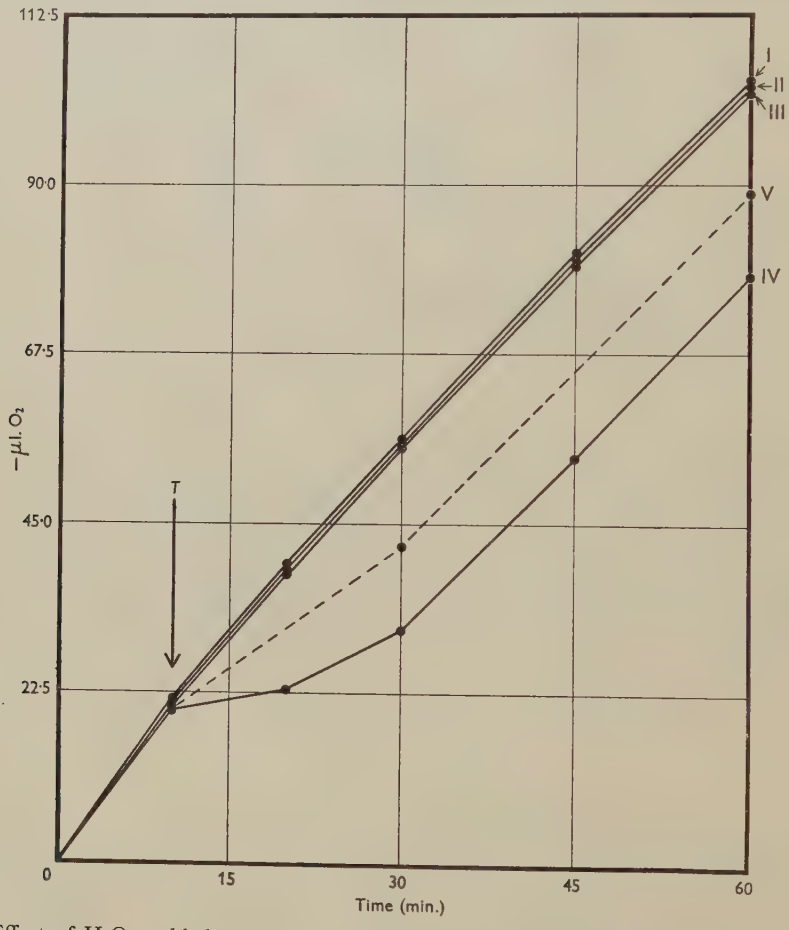

Fig. 2. Effect of H_2O_2, added at *T*, on O_2 uptake of sea-urchin spermatozoa, *Echinus esculentus*. I, control; II, final concentration of H_2O_2, $7{\cdot}8 \times 10^{-5}$M; III, final concentration of H_2O_2, $3{\cdot}9 \times 10^{-5}$M; IV, final concentration of H_2O_2, $4{\cdot}3 \times 10^{-4}$M; V, curve IV, corrected for evolution of O_2. Number of sperm in manometers, $1{\cdot}17 \times 10^9$.

the activity of '2nd' seminal plasma (see Table 3, V_3). Both experiments suggest that in certain conditions, catalase diffuses out of spermatozoa, or off their surfaces, into the medium. This would be of great interest if the process of centrifugation and re-suspension did not damage or kill a significant number of spermatozoa. The only quantitative test for injury or lack of injury at present available is that of O_2 uptake; there is no doubt that O_2 uptake is markedly reduced by centrifugation.

Effect of hydroxylamine on catalatic activity of semen and seminal plasma. 10^{-4}M-hydroxylamine strongly inhibited the decomposition of H_2O_2 by semen and seminal plasma.

Catalatic activity of sea water. When H_2O_2 was added to sea water there was no evolution of O_2.

Effect of hydrogen peroxide and hydroxylamine on O_2 uptake. The effect of H_2O_2 is shown in Fig. 2. In this experiment, measurements of O_2 uptake were made on

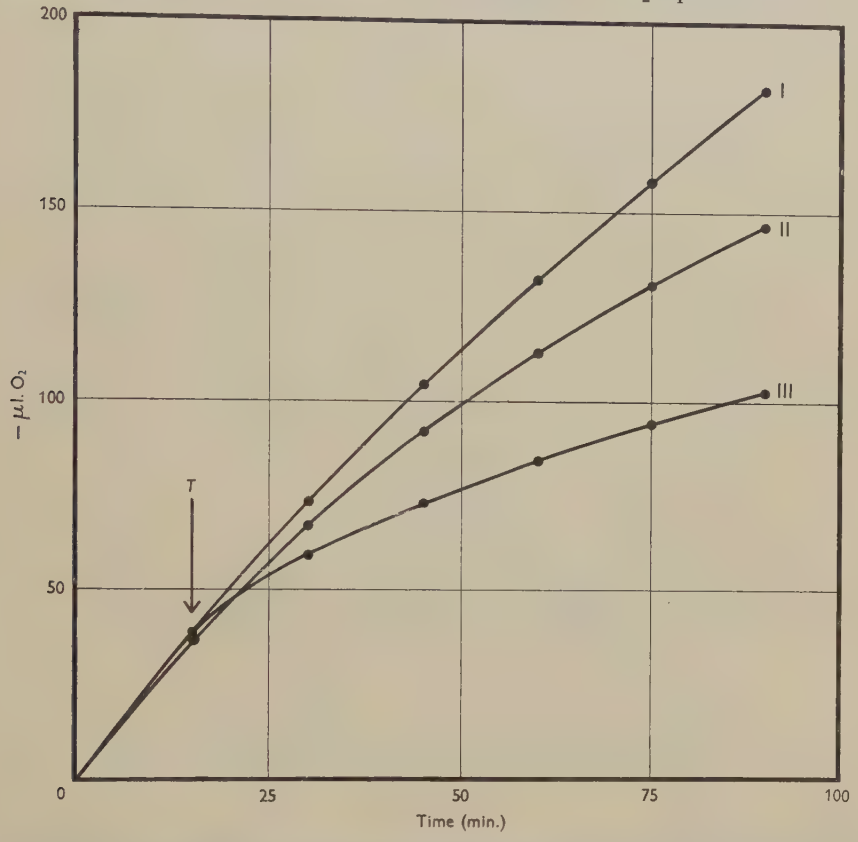

Fig. 3. Effect of neutralized hydroxylamine hydrochloride, added at *T*, on O_2 uptake of sea-urchin spermatozoa, *Echinus esculentus*. I, control; II, final concentration of hydroxylamine, $3{\cdot}31 \times 10^{-4}$M; III, final concentration of hydroxylamine, $1{\cdot}66 \times 10^{-4}$M. Number of sperm in manometers, $1{\cdot}22 \times 10^{9}$.

four identical sperm suspensions at the same time. After 10 min., during which the four suspensions respired at the same rate, 0·3 ml. sea water was added to the sperm suspension in the first vessel, curve I; 0·3 ml. sea water containing H_2O_2 to the suspension in the second vessel, final concentration $7{\cdot}8 \times 10^{-5}$M, curve II; 0·3 ml. sea water containing H_2O_2 to the suspension in the third vessel, final concentration $3{\cdot}9 \times 10^{-5}$M, curve III; and 0·3 ml. sea water containing H_2O_2 to the suspension in the fourth vessel, final concentration $4{\cdot}3 \times 10^{-4}$M, curve IV. The spermatozoa were able to deal with the added H_2O_2 in each case, though in the case of the highest

concentration of H_2O_2, there was a pronounced shock effect, from which the spermatozoa only recovered after 20 min., as evidenced by the rate of O_2 uptake becoming similar to that in the control. Part of the depressed O_2 uptake in curve IV is due to the positive pressure in the manometer, when the added H_2O_2 is decomposed into O_2 and H_2O. This positive pressure does not account for the whole of the drop in O_2 uptake, as curve V, which is curve IV corrected for the simultaneous evolution

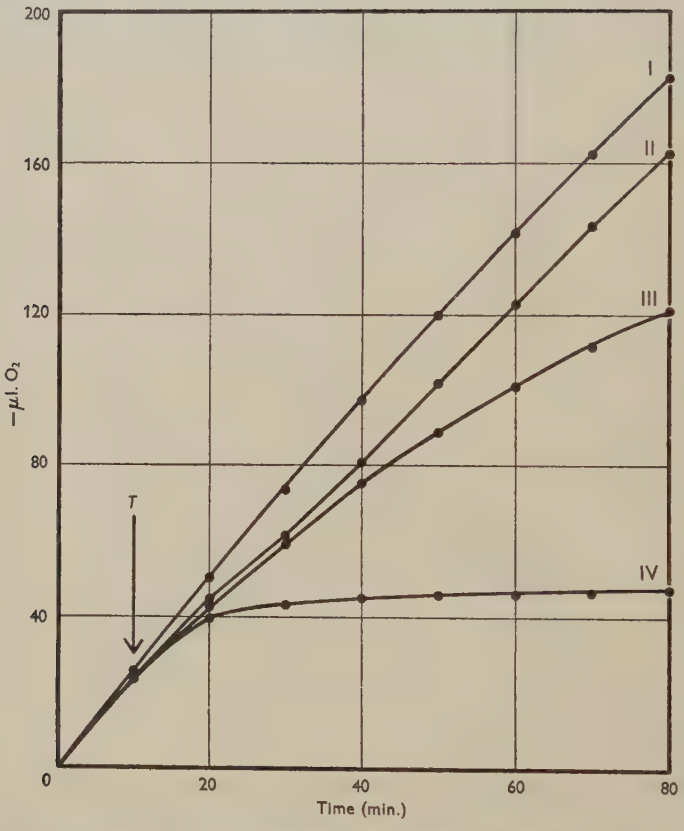

Fig. 4. Effect of hydroxylamine, H_2O_2, separately and together, added at *T*, on O_2 uptake of sea-urchin spermatozoa, *Echinus esculentus*. I, control; II, final concentration of H_2O_2, $1{\cdot}2 \times 10^{-4}$M; III, final concentration of hydroxylamine, $3{\cdot}31 \times 10^{-4}$M; IV, final concentrations of H_2O_2 and hydroxylamine, $1{\cdot}2 \times 10^{-4}$ and $3{\cdot}31 \times 10^{-4}$M. Number of sperm in manometers, $8{\cdot}90 \times 10^8$.

and uptake of O_2, shows. The amount of O_2 evolved in the Exps. II and III is too small to be detected.

Hydroxylamine depresses O_2 uptake (Fig. 3) *and* motility. O_2 uptake was measured in three identical sperm suspensions which respired at the same rate before treatment. After 15 min., 0·3 ml. sea water was tipped into the first suspension, curve I; hydroxylamine, in sea water, final concentration $3{\cdot}313 \times 10^{-4}$M, was tipped into the second suspension, curve II; and hydroxylamine in sea water, final concentration $1{\cdot}66 \times 10^{-4}$M was tipped into the third suspension, curve III. Apart from affecting

O_2 uptake, the stronger hydroxylamine solution markedly reduced the motility of the spermatozoa.

At such low concentrations, hydroxylamine is more likely to inhibit catalase than cytochrome. The cytochrome system of sea-urchin spermatozoa, might, however, be unusually sensitive to hydroxylamine; in order to exclude this interpretation of the

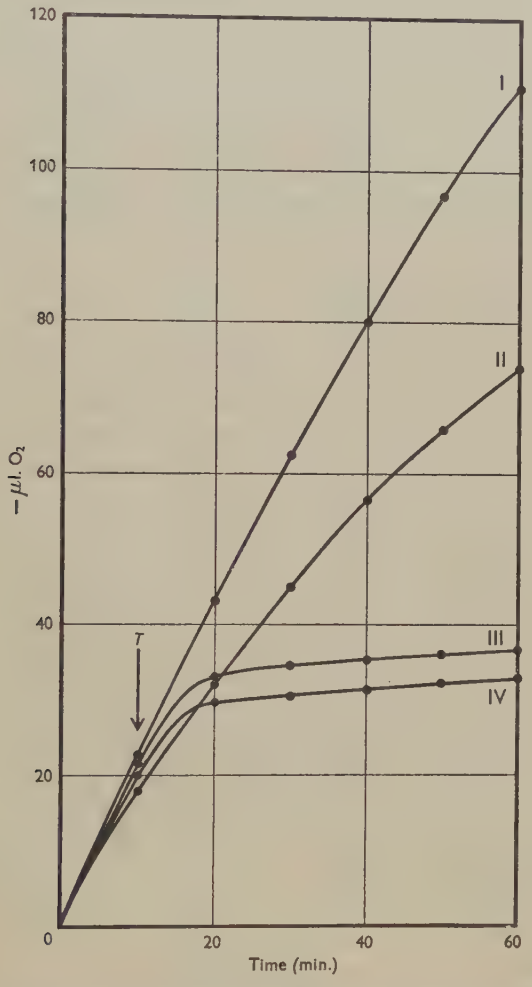

Fig. 5. Comparison of O_2 uptake after addition, at *T*, of lethal amounts of H_2O_2, alone and with hydroxylamine. I, control; II, final concentration of hydroxylamine, $3{\cdot}31 \times 10^{-4}$ M; III, hydroxylamine, final concentration $3{\cdot}31 \times 10^{-4}$ M and H_2O_2, final concentration $2{\cdot}9 \times 10^{-4}$ M. IV, H_2O_2, final concentration $2{\cdot}9 \times 10^{-4}$ M. Number of sperm in manometers, $1{\cdot}1 \times 10^9$.

observed inhibition, experiments were done to test the effect of hydroxylamine, H_2O_2 and a combination of both, on the O_2 uptake of sperm suspensions. The results are shown in Fig. 4. Curve I is the control in which at $t = 10$ min., 0·30 ml. sea water was tipped into the sperm suspension in the main vessel. Curve II shows the effect of adding H_2O_2, final concentration $1{\cdot}2 \times 10^{-4}$ M. The small positive

pressure due to the evolution of O_2, or the 'shock' effect of adding H_2O_2, is clearly visible between $t=20$ and $t=30$. After this, recovery is complete, showing that the spermatozoa were unaffected by the added H_2O_2. Curve III shows the characteristic reduction in O_2 uptake on addition of hydroxylamine, final concentration $3{\cdot}31 \times 10^{-4}$M. Curve IV shows the effect of adding the two reagents simultaneously, the final concentrations being the same, that is, H_2O_2, $1{\cdot}2 \times 10^{-4}$M; and hydroxylamine, $3{\cdot}31 \times 10^{-4}$M. The dramatic and abrupt cessation of O_2 uptake and motility which follows this treatment could be interpreted as showing that at these concentrations hydroxylamine specifically inhibits catalase activity and not the cytochrome system in the spermatozoa. For in the presence of hydroxylamine, a concentration of H_2O_2, which is harmless alone, is extremely toxic. The possibility that in these conditions, hydroxylamine exerts a different and perhaps indirect effect cannot be completely excluded. Both in curves II and IV there is a significant delay before the H_2O_2 exerts its maximum effect, as in the experiments on the catalatic activity of spermatozoa in sea water.

On the basis of these experiments we should expect to be able to differentiate manometrically between a lethal dose of H_2O_2 and the same dose of H_2O_2 + hydroxylamine; the apparent total O_2 uptake should be lower in the former than in the latter, because of the evolution of O_2, when the catalase is not inactivated. An experiment to test this is shown in Fig. 5. Curve I is the control in which 0·3 ml. sea water was added to the sperm suspension in the main vessel at $t=10$. In curve II hydroxylamine in 0·3 ml. sea water was added, the final concentration being $3{\cdot}31 \times 10^{-4}$M. In curve III, hydroxylamine, final concentration $3{\cdot}31 \times 10^{-4}$M, and H_2O_2, final concentration $2{\cdot}9 \times 10^{-4}$M, were added simultaneously. In curve IV 0·3 ml. sea water, containing H_2O_2, final concentration $2{\cdot}9 \times 10^{-4}$M, was added at $t=10$, the total O_2 consumed by the sperm in the presence of H_2O_2 alone was apparently about 4 μl. less than when H_2O_2 and hydroxylamine were added together.

Volume of a spermatozoon. The volume of a sea-urchin spermatozoon is about 15 μ^3. This figure is only approximate and is obtained by graphical integration of drawings based on microphotographs. It assumes that the sea-urchin spermatozoon, unlike that of the bull, has axial symmetry, i.e. that the correct volume of the spermatozoon is obtained by estimating the volume of the solid formed by rotating the spermatozoon about its long axis.

DISCUSSION

Existence of catalase in spermatozoa and seminal plasma. The catalatic activity of seminal plasma in phosphate buffer is about twice that of semen in the same medium. The experiments on the increase in seminal catalatic activity in distilled water, and those showing that washed semen does not lose its ability to decompose H_2O_2 prove without reasonable doubt that both sea-urchin spermatozoa and seminal plasma contain catalase. The question can, however, be examined from an entirely different point of view, by considering the relative volumes of sperm and seminal plasma in a sample of known catalatic activity and sperm density. In the experiment in Table 3, there were $2{\cdot}85 \times 10^{10}$ sperm/ml. semen. The volume of a spermatozoon

is about 15 μ^3. 0·30 ml. semen therefore consisted of 0·128 ml. spermatozoa and 0·172 ml. seminal plasma. 0·30 ml. seminal plasma decomposed 52 μl. O_2 from an H_2O_2 solution containing 155 μl. O_2 in 1 min. 0·172 ml. seminal plasma would therefore have decomposed 30 μl. O_2 in the same time. But in fact this amount of seminal plasma, in the presence of 0·128 ml. spermatozoa, produced 92 μl. O_2 in 1 min., showing that the sperm contributed to the catalatic activity of the semen.

Mention has already been made of the difficulty, if not the impossibility of measuring the catalatic activity of spermatozoa as opposed to semen or seminal plasma. It is therefore equally difficult quantitatively to relate the catalatic activity of semen and washed spermatozoa. Table 3 suggests that if it were possible to measure it, the catalatic activity of spermatozoa might well be much higher than that of seminal plasma. It is evident from this table that the catalatic activity of washed sea-urchin spermatozoa requires further study, though such a study presents several difficulties.

Influence of H_2O_2 *and hydroxylamine on* O_2 *uptake.* The low concentration of hydroxylamine which affects the O_2 uptake and motility of sea-urchin spermatozoa suggests that, in these conditions, catalase rather than cytochrome is attacked by this inhibitor. This view is strengthened by the observation (Rothschild, 1948*b*) that a large reduction in O_2 uptake can be effected by CO in the dark, which prevents the reoxidation of cytochrome oxidase, without motility being correspondingly reduced. This indicates that, although sea-urchin sperm motility is dependent on the presence of O_2, only part of the O_2 consumption of the spermatozoa is directly concerned with the energy requirements of movement. Hydroxylamine, on the other hand, reduces motility even when it inhibits O_2 uptake to a relatively small extent. If the view is accepted that at low concentrations hydroxylamine combines with catalase, this observation shows that catalase has a functional role in sea-urchin sperm metabolism. The experiments showing that H_2O_2 is harmless by itself but highly toxic in the presence of hydroxylamine prove the first point, that the effect of hydroxylamine in these conditions is to inactivate catalase; they do not unequivocally prove that sperm metabolic processes normally result in the production of H_2O_2 which is decomposed by catalase. Catalase may have some role other than decomposing H_2O_2; if this is so, when this role is interfered with by hydroxylamine, O_2 uptake and motility may be reduced for some reason other than the accumulation of H_2O_2. Even if this is true, catalase has been shown to be available for the decomposition of H_2O_2 as well as to perform this other hypothetical function. Though there is a case for the view that the function of catalase in sea-urchin spermatozoa is to decompose H_2O_2 formed during metabolism, the only way of proving this explicitly would be by a chemical demonstration that H_2O_2 accumulates in the presence of hydroxylamine.

Evans (1947) observed that heavily irradiated sea water harmed the spermatozoa of *Arbacia punctulata*. His interpretation was that the irradiation produced H_2O_2 which, at concentrations of less than 0·5 part per million, was toxic to the spermatozoa. This result might be considered surprising in view of the catalase content of sea-urchin spermatozoa, which Evans also observed (see Lwoff & Morel, 1942).

A further surprising feature of these experiments was that the toxic effects of H_2O_2 were neutralized both when catalase *and* heat-inactivated catalase were added to the suspensions. The differences between Evans's results and those described here may in part be due to differences in technique. In one case fertilizing capacity and survival times were observed; in the other O_2 uptake was measured. There are certain disadvantages in adding H_2O_2 to manometers which are subsequently shaken, but the experiments described in this paper are not affected by them, because the H_2O_2 was not decomposed in control experiments without spermatozoa and because the H_2O_2 experiments were combined with ones in which hydroxylamine was added at the same time to inhibit catalase, when the toxic action of H_2O_2 was plainly visible.

From the data in the Results section, a rough calculation can be made of the number of catalase molecules per spermatozoon. According to Green (1940), one mole of catalase decomposes $4{\cdot}2 \times 10^4$ moles of H_2O_2 per second at 0° C. As from Table 3, $8{\cdot}55 \times 10^9$ washed spermatozoa produced 90 μl. O_2 in 1 min., each spermatozoon contains less than 500 molecules of catalase.

APPENDIX

Catalatic activity of human blood

Dry weight of 1 ml. blood (R), 0·2293 g.

Dilution of blood for manometric experiment, 1 ml./1667 ml. neutral phosphate buffer.

Main vessel, 4·0 ml. blood phosphate mixture.

Dangling cup, 0·1 ml. H_2O_2 solution, containing 146 μl. O_2.

O_2 produced in 1 min. after mixing, 87 μl.

A_c, 6800.

SUMMARY

1. Spermatozoa and seminal plasma of *Echinus esculentus* contain catalase.

2. At 15° C., 4 ml. of a suspension of semen diluted with neutral phosphate buffer in the ratio 1:13 produced in 1 min. 90 μl. O_2 from an H_2O_2 solution containing 150 μl. O_2. The dry weight of semen in the suspension was 45 mg. and the number of spermatozoa $8{\cdot}55 \times 10^9$. Under the same conditions, seminal plasma obtained by centrifuging semen produced 50 μl. O_2 in 1 min. The dry weight of seminal plasma in the suspension was 12 mg. Human blood, dry weight 229·3 mg./ml., must be diluted with phosphate buffer in the ratio 1:1700 to produce the same amount of O_2 in 1 min. as the above suspension of semen. If catalatic activity is defined by the equation $A_c = (gt)^{-1} \ln \{a/(a-x)\}$, where g = weight in g./ml. of the catalase-containing material, t = 1 min., a = initial substrate concentration (H_2O_2), and x = amount of H_2O_2 decomposed in 1 min. at 15° C., A_c = 80–100, 150–200 and 6800 respectively for sea-urchin semen, sea-urchin seminal plasma and human blood.

3. The catalatic activity of semen and seminal plasma is strongly inhibited by hydroxylamine.

4. The O_2 uptake and motility of sea-urchin spermatozoa is unaffected by M/5000 H_2O_2. Higher concentrations of H_2O_2, M/3000–5000, produce a pronounced 'shock' effect, from which the spermatozoa often completely recover.

5. Low concentrations of hydroxylamine, M/3000, reduce O_2 uptake and motility.

6. Sea-urchin spermatozoa are almost instantly killed by combinations of hydroxylamine and H_2O_2, at concentrations which are relatively innocuous when the substances are added separately.

7. A rough calculation indicates that a single spermatozoon contains less than 500 molecules of catalase.

8. A new method of adding H_2O_2 to catalase-containing material in a manometer is described.

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A NOTE ON THE CATALASE CONTENT OF SEA-URCHIN EGGS

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(With One Text-Figure)

During experiments on the catalase content of sea-urchin spermatozoa, opportunities arose of measuring the catalatic activity of sea-urchin eggs. Fertilized and unfertilized eggs of *Echinus esculentus* were examined, the jelly having been removed in each

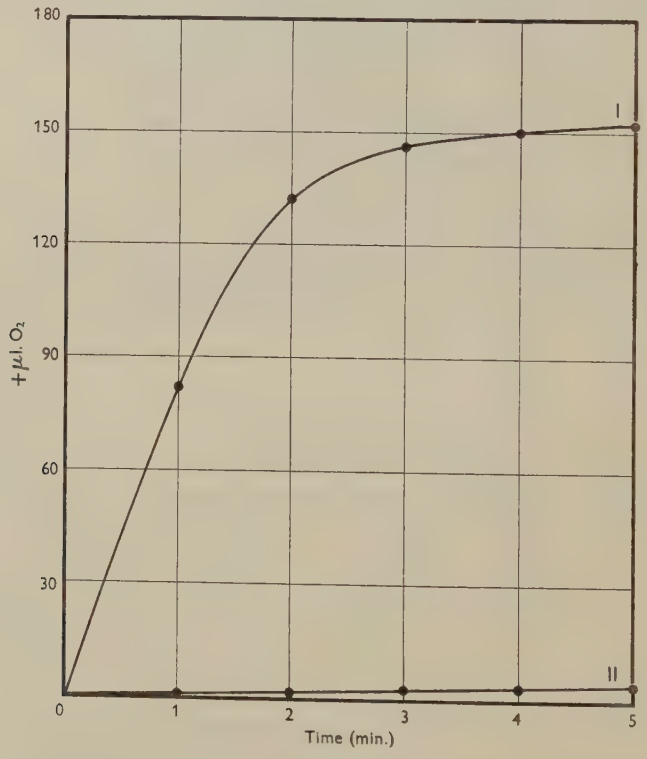

Fig. 1. Effect of neutralized hydroxylamine hydrochloride, final concentration $9{\cdot}3 \times 10^{-5}$M, on catalatic activity of unfertilized eggs of *Echinus esculentus*. In each curve the manometer contained 12·44 mg. dry weight of eggs in 4·1 ml. neutral M/40 phosphate buffer. I, O_2 evolution (control); II, O_2 evolution in presence of hydroxylamine. $T°$ C., 15. Shaker, 170 c.p.m. 0·1 ml. H_2O_2 solution contained 152 μl. O_2, added at $t=0$.

case. Holter & Linderstrøm-Lang (1936) and Holter (1937) investigated the catalase content of sea-urchin eggs, but they were particularly concerned with the location

of this enzyme in the cell. The catalatic activity of sea-urchin eggs has attracted attention on several occasions in the past (see, for example, Kobert, 1903), but the results were unquantitative and sometimes contradictory. Furthermore, no workers examined the effect of low concentrations of hydroxylamine on the apparent catalatic activity of these cells. Hydroxylamine is a specific inhibitor of catalase as opposed to other substances which catalytically decompose H_2O_2 into O_2 and H_2O.

The experimental procedure was the same as that described elsewhere in this *Journal* (Rothschild, 1950). Twenty-five determinations were made on unfertilized eggs at 15° C.; the mean catalatic activity, A_c (see immediately preceding paper) was 660. The comparable figure for sea-urchin semen is 90. Unfertilized sea-urchin eggs are notoriously variable in their physiological properties. These experiments were not exceptional in this respect, different batches of eggs showing considerable variability in regard to catalase content. The estimated standard error of the mean was 180. Five comparisons of fertilized and unfertilized eggs were also made, involving twenty-three measurements. The difference between fertilized and unfertilized eggs was insignificant, though in all comparisons the catalatic activity of fertilized eggs was slightly lower than that of unfertilized ones.

Low concentrations of neutralized hydroxylamine hydrochloride almost completely inhibit the catalase activity of the eggs. Fig. 1, which illustrates this, would apply equally well to sea-urchin spermatozoa, both as regards the form of the O_2 evolution curve and the inhibition of O_2 evolution by hydroxylamine.

Measurements of catalase content cannot be made on eggs in sea water. When an attempt is made to do this, there appears to be a larger evolution of O_2 than is theoretically available in the H_2O_2 added to the egg suspensions. The H_2O_2 and the high rate of shaking cytolyse the eggs which produce acid. This decomposes the bicarbonate in the sea water, the evolved CO_2 causing a positive pressure in the manometer, in addition to that due to O_2 evolution.

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SEASONAL CHANGES IN BLOOD SUGAR, FAT BODY, LIVER GLYCOGEN, AND GONADS IN THE COMMON FROG, *RANA TEMPORARIA*

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(*Received* 18 *May* 1949)

(With Ten Text-figures)

INTRODUCTION

In recent years a large volume of research, mainly on the Mammalia, has shown the important role played by the endocrine glands in the general control of carbohydrate metabolism and in the regulation of the blood-sugar level. The work in this field has been comprehensively reviewed by Cori (1931), Soskin (1941), and Soskin & Levine (1946). The extent to which a similar endocrine control might be operative in lower vertebrates, such as the Amphibia, has not been so extensively investigated. The isolated frog's liver has been used by several workers to test the action of various hormones on liver glycogenolysis (Lesser, 1920; Issekutz, 1924; Fluch, Greiner & Loewi, 1935; Kepinov, 1937). Houssay and his co-workers (Houssay & Biassotti, 1931, 1933; Houssay, Biassotti & Rietti, 1934) have shown that the anterior pituitary in a toad, *Bufo arenarum*, exerts a 'diabetogenic' action, and the severity of the diabetes resulting after pancreatectomy is greatly ameliorated if the anterior lobe of the pituitary is removed. Slome (1936) also found that the blood sugar of *Xenopus laevis* was reduced after anterior lobe hypophysectomy. No reference has been found in the literature to any physiological work on seasonal changes in the activity of the endocrine glands of the frog, though Sklower (1925) has described seasonal differences in the histological appearance of various glands. Aron & Schwartz (1925) and Aron (1926) demonstrated the occurrence of seasonal histological changes in the Islets of Langerhans in the pancreas and in the interstitial tissue of the testis. Smith (1938) was able to correlate the latter with the increasing intensity of the induced clasping action of the male during the autumn and winter.

In contrast with the rather incomplete picture of the role of the endocrine glands in amphibian metabolism, there is a good deal of information available regarding the nature and extent of certain seasonal changes which occur in the frog (Holmes, 1927; Holzapfel, 1937). Among the organs showing well-defined changes are the liver, fat bodies, and the gonads. Athanasiu (1899) and Pflüger (1907) first reported the occurrence of marked seasonal changes in the glycogen content of frogs, and their work has subsequently been confirmed by Kato (1910), Bleibtreu (1911), and Goldfederowa (1926). All these authors are agreed that the liver glycogen and total body glycogen of the frog attain a high maximum value in the autumn, and fall to

a minimum in the spring. It was considered that the physiological mechanism controlling this seasonal change in glycogen content might prove to be of general interest if it could be elucidated. By analogy with the conditions found in the higher vertebrates it would seem probable that the endocrine system would be involved in such metabolic changes, and that there would be a reasonable expectation of associated changes in the post-absorptive blood-sugar level. The literature provides several references to estimations of blood sugar in the frog, but some of these relate to one particular season only (Bang, 1913; Lesser, 1913; Slome, 1936), while in those cases where the observations extend over a few months the duration and conditions of captivity are not always well defined (Besson, 1945; Scott & Kleitman, 1921). As a first approach to the problem it was therefore decided to carry out a seasonal survey to investigate the possible occurrence of correlations between the changes in the post-absorptive blood-sugar level, the development of the fat bodies, the glycogen content of the liver, and the development of the gonads.

MATERIAL AND METHODS

As the primary object of the present work was the investigation of the occurrence of seasonal variations in the post-absorptive blood sugar, it was necessary to standardize the sampling procedure so that all the samples should be comparable. To ensure this, interference by alimentary factors also had to be avoided as far as possible. From preliminary observations it was found that frogs examined after being kept in captivity for 48 hr. without access to food, in many cases still had partly digested food in the stomach. On examination after 72 hr., however, the stomach was usually found to be empty, and in the few exceptional cases digestion was far advanced. This agrees with the work of Frost (1932), who found that food required 48 hr. to pass through the gut of *Rana pipiens*. Hill (1911) has shown that the heat production of frogs falls considerably in the first 15 days of captivity without food. This decline in metabolic rate due to captivity and inanition raised the question of whether the blood-sugar level might also be affected. It was for this reason considered advisable to deal with the samples after the minimum time in captivity necessary to avoid errors due to alimentary hyperglycaemia. The third day after capture was finally selected as the time for taking all blood samples, and unless otherwise stated all blood-sugar values in this paper relate to frogs which have been in captivity without feeding for approximately 72 hr.

Most of the frogs used in this work were obtained from a collector in Shropshire. They were received in the laboratory 24 hr. after capture, and at once put in a glass-sided aquarium tank which was slightly tilted and contained enough water to form a pool at one end, while leaving the other end dry. The slate bottom of the tank provided a dark background, and all the frogs used for the routine observations were therefore dark-ground adapted. Slome (1936) has reported a difference in blood sugar between dark- and light-ground adapted *Xenopus laevis*. While no indication of such a difference was found in preliminary observations made by the author (*vide infra*), it was nevertheless thought advisable to standardize the background conditions. The room in which the frogs were kept was unheated and well ventilated,

the temperature being approximately atmospheric. At times during the summer samples of frogs were obtained locally, these being used as parallel samples for comparison with those from the usual source. In no case were any significant differences found and the results have not been shown separately.

A standard procedure was adopted for dealing with each frog in the samples. The frog was removed from the tank with the minimum of disturbance, care being taken to prevent the frog's struggling, and immediately pithed. The heart was quickly exposed and the blood sample withdrawn from the ventricle. The liver was then excised, blotted between filter-paper to remove the excess moisture, rapidly weighed and transferred to a centrifuge tube containing hot 40% caustic potash. The frog itself was then weighed, after which the fat bodies and, in some samples, the gonads were removed, blotted and weighed. The blood samples were obtained from the ventricle by means of a hypodermic syringe which had previously been rinsed out with a dilute solution of potassium oxalate and air dried. The volume of blood obtainable by this method varied considerably, but usually from 0·3 to 0·4 ml. could be drawn off quite rapidly. The blood was transferred to an oxalated crucible from which a 0·1 ml. sample was pipetted into the zinc hydroxide protein coagulant. The sugar content of these samples was estimated by the method of Hagedorn & Jensen (1923). Duplicate analyses were made on numerous occasions, and the final burette readings never differed by more than 0·01 ml. Liver glycogen was estimated by Sahyun's (1931) method, the glucose content of the digest after hydrolysis being determined on an aliquot sample by reduction of potassium ferricyanide as in the blood-sugar estimations. The glycogen found was expressed as a percentage of the wet weight of the liver. The fat body, liver and gonad weights were expressed as percentages of the total body weight.

Samples of frogs were obtained every month (with the exception of February) from January 1948 to January 1949. The number in each sample varied from 12 to 26, the average sample number for the whole series being 16. The weight of the frogs used usually lay between 20 and 25 g., the extreme range being from 12 to 50 g. (usually gravid females). The lower size limit was set by the difficulty of obtaining blood samples from small frogs. At the lowest size used all the frogs had attained sexual maturity.

The seasonal changes in fat bodies, gonads, liver weight, and liver glycogen content

(*a*) *Fat bodies*

The cycle of fat-body development found in 1948 is shown in Fig. 1. If a true comparison between the sexes is to be made it is necessary to make allowance for the differential development of the reproductive organs, for, as March (1937) has pointed out, the great development of the ovaries and oviducts causes a disproportionate increase in the body weight of the female. Therefore the weight of the reproductive organs was deducted from the total body weight of the females in the July to January samples, inclusive, before calculating the percentage weight of the fat body. As the testes represent a significant proportion of the body weight in

August, their weight was similarly deducted from the total male body weight in the July, August and September samples to achieve uniformity of treatment. At other times of the year the correction for testis weight is insignificant. Only in January 1949 was a significant sex difference found, and this is the only month when the fat-body weights have been shown by sexes in Fig. 1.

Previous workers have found a very similar cycle for the changes in the fat bodies of frogs (Gaule, 1901; Victoroff, 1908; Kennel, 1912). The fat body attains its

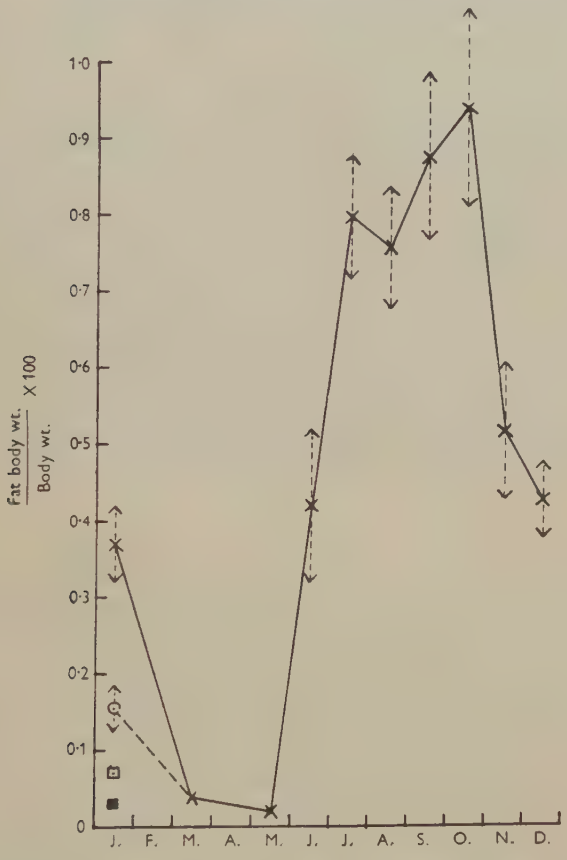

Fig. 1. Seasonal variation in weight of fat body of *R. temporaria* in 1948. ⊙, females Jan. 1949; ⊡, sexually active males Jan. 1948; ■, sexually active females Jan. 1948. Vertical broken lines show the standard errors of the sample means.

maximum development in October, this being followed by a decrease through the winter culminating in a rapid fall to a minimum at the spawning season. The effect of sexual activity on the fat body is shown by the difference between the two January samples. All the frogs received in January 1948 were in amplexus, while those in January 1949 were not sexually active, and it can be seen that the fat body had not been so extensively depleted in the latter group. There is some conflict in the literature as to the existence of a sex difference in the fat-body development in the autumn. Kennel (1912) found the female fat body in *Rana temporaria* to be con-

siderably larger than that of the male from June to March. Gaule (1901), however, found it to be better developed in the male *R. esculenta* from September to June. In the present work, where a correction has been made for the weight of the reproductive organs, a significant sex difference was found only in January ($P < 0.01$), when the male fat body was the larger. This is perhaps an indication that the female draws more extensively on the stored reserves of nutriment during the winter than the male. It may be noted that there was always a wide range of individual variation in fat-body weight, as can be seen from the standard errors of the means plotted in Fig. 1, and much larger sample numbers would have to be used before a reliable test of possible sexual differences could be made.

The most striking feature of the present results is the occurrence of a very rapid regeneration of the fat body in June and July. Eleven frogs caught on 31 May showed no difference in fat-body condition from the post-spawning samples, but examination of thirteen frogs caught on 15 June revealed that fat-body regeneration was well under way. The average fat-body weight for the last group was 0·42% of the body weight compared with 0·02% for those caught at the end of May. This rapid increase was continued in the July sample, but a definite check was seen in August. A second small increase was found in September and in October when the maximum for the year was attained. Gaule (1901) found a similar rapid increase in fat-body weight for *R. esculenta* during July. In the early part of the month the fat body represented 0·03% of the body weight, compared with 0·88% at the end of the month.

(*b*) *Gonads*

The seasonal changes in weight of the gonads are shown in Fig. 2. The data on which these curves are based were not obtained from the samples examined in 1948 as the gonads were not weighed in all cases, but were obtained by the author in 1934 (previously unpublished). When average gonad weights were determined in 1948 they showed no significant departures from the corresponding 1934 records. The general cycle of gonad development agrees closely with that found by other workers (Holmes, 1927). The most obvious change in the testis weight was the sharply defined maximum in August accompanying spermatogenesis. In late July the testis weight was 3·2%, in August it reached 6·0%, and by September it had fallen again to 3·1% of the body weight. During the winter there is a slight decrease in weight, with a more obvious fall at the spawning period due probably to the transfer of spermatozoa to the vesicula seminales. The regeneration of the ovaries is a more extended process. They show a gradual increase in weight during July and August, followed by a more rapid rise to November when the maximum weight is nearly attained. The ovarian weight shows a steep fall at the spawning season when ovulation takes place.

(*c*) *Liver weight*

The variation in liver weight during the year is shown in Fig. 3. As in the case of the fat body adjustment has been made where necessary for the disproportionate

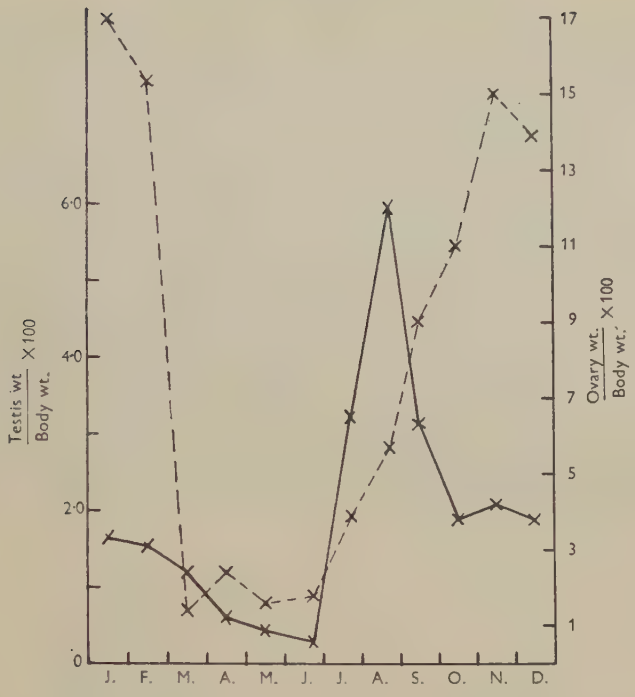

Fig. 2. Seasonal variation in weight of the gonads of *R. temporaria* in 1934. ×———×, testis; ×– – – –×, ovary.

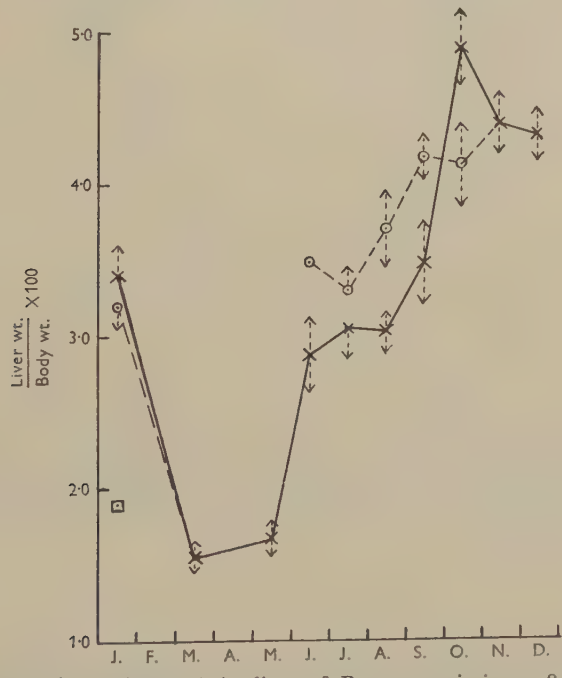

Fig. 3. Seasonal variation in weight of the liver of *R. temporaria* in 1948. ×———×, males; ⊙– – – –⊙, females; ⊡, sexually active males Jan. 1948. Vertical broken lines show the standard errors of the sample means.

increase in the reproductive organs in the two sexes. The weight of the ovaries and oviducts or of the testes has been deducted from the total body weight in the same way and for the same months as detailed in the fat-body section. March (1937) found that the female liver weight always exceeded that of the male when adjustment was made for the weight of the reproductive organs. The present results confirm this with the exception of those for October and January when the male liver was the heavier. The standard errors of the means are, however, too large for any statistical significance to be attached to these differences. The influence of the onset of sexual activity on liver weight is shown by the mean value given by the January 1948 frogs, which were all in amplexus. The mean liver weight of the males in this sample was 1·9% of the body weight compared with 3·4% in January 1949 when the frogs were not sexually active. Apart from the sex difference there was a regular cycle of liver-weight change. The minimum occurred during and after the breeding season in March, April and May. The first obvious post-spawning increase took place in June to be followed by a further rise in the autumn, the maximum for the year being reached in October.

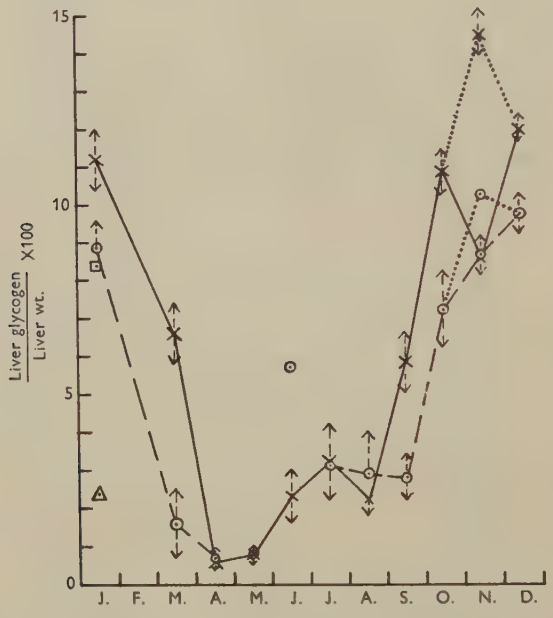

Fig. 4. Seasonal variation in liver glycogen, calculated as a percentage of the wet weight of the liver, of *R. temporaria* in 1948. ×———×, males; ⊙– – –⊙, females; ⊡, sexually active males Jan. 1948; △, sexually active females Jan. 1948. Dotted lines in November show the liver glycogen content found after 4 days in captivity. Vertical broken lines show the standard errors of the sample means.

(*d*) *Liver glycogen*

The monthly means of the estimations of the glycogen content of the liver are shown in Fig. 4 as percentages of the wet weight of the liver. In view of the fact, however, that the relative weight of the liver itself has been shown to fluctuate

seasonally, it was considered that these results would more accurately depict the seasonal changes in glycogen storage if they were expressed as liver glycogen contained in 100 g. of total body weight of frog. The data when recalculated in this way are shown in Fig. 5, where, as in the case of the fat-body and liver weights, the weight of the reproductive organs has been deducted from the total body weight for the same months previously detailed.

In the main the changes in liver glycogen follow a similar cycle to that just described for the liver weight. There is a post-spawning minimum in April and May, followed by a slight increase in June. The June sample contained only three female frogs, which showed liver glycogen contents of 7·6, 7·2 and 2·3% of the wet weight of the liver (mean $5{\cdot}7 \pm 1{\cdot}7\%$). In view of the small sample number and large

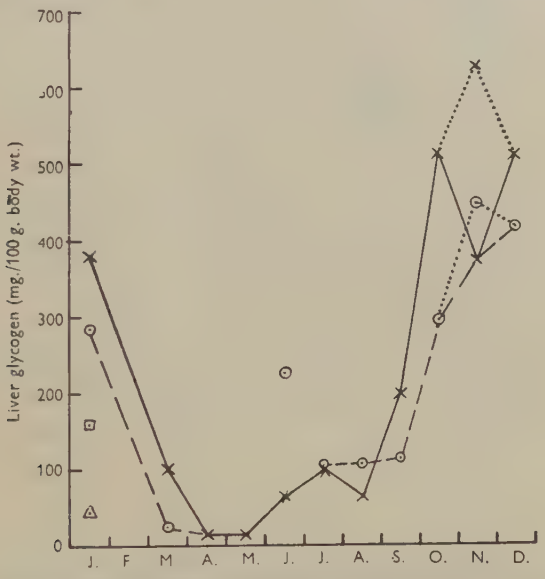

Fig. 5. Seasonal variation in liver glycogen, calculated as mg. of liver glycogen per 100 g. of frog. For explanation of symbols, see legend to Fig. 4.

variation this mean value for female liver glycogen has been shown as an isolated point in Figs. 4 and 5. It is considered that this transient rise in female liver glycogen shown by this sample is probably due to sampling errors. In July and August there is little further change, but in the male there is a rapid increase in September, while in the female this autumn increase is delayed until October and is not as great as that of the male. The points shown joined by dotted lines in Figs. 4 and 5 relate to glycogen values found after frogs from the same sample had been in captivity for 4 days. They show that a significant increase in glycogen content had taken place, especially in the male, in the 24 hr. since the normal 72 hr. observations were made. This phenomenon is referred to again below (p. 428). There is a gradual fall in liver-glycogen content during the winter, largely owing to the decrease in liver weight. A sharper fall occurs at the spawning season, the female liver glycogen being depleted more rapidly than that of the male. The influence of sexual activity

is again well shown by the sexually active sample received in January 1948. This annual cycle is similar to that described by previous authors (Athanasiu, 1899; Pflüger, 1907; Kato, 1910; Bleibtreu, 1911; Goldfederowa, 1926).

Seasonal changes in blood sugar

The annual cycle of variation of the sugar content of the blood of frogs kept in captivity for 72 hr. is shown in Fig. 6. It can be seen that during the greater part of the year the blood sugar did not depart very far from a value of 40 mg./100 ml., with

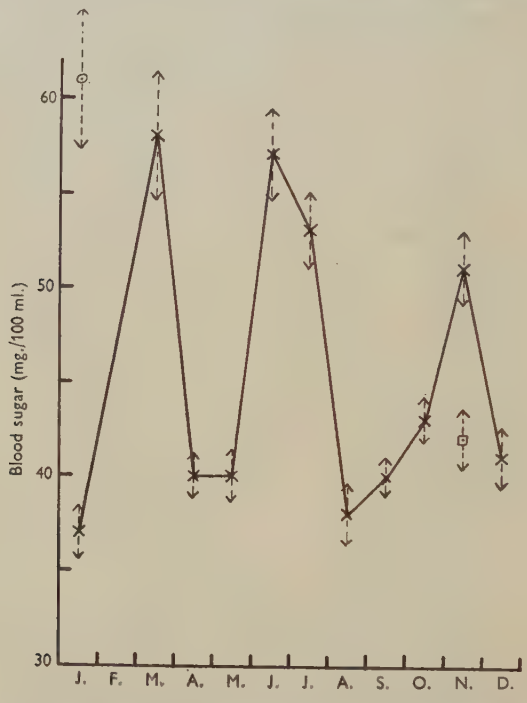

Fig. 6. Seasonal change in blood sugar of *R. temporaria* during 1948, after 3 days in captivity without food. ⊙, sexually active sample Jan. 1948; ⊡, after 4 days in captivity in Nov. 1948. Vertical broken lines show the standard errors of the sample means.

the exception of three periods when a significant increase was found. The first of these occurs at the spawning period, the mean blood sugar in March being $58 \pm 3{\cdot}5$ mg./100 ml. The correlation between this high blood sugar and sexual activity is indicated by the high mean of $61 \pm 3{\cdot}7$ mg./100 ml. given by the sexually active sample in January 1948, while the quiescent sample received in January 1949 gave a mean value of only $37 \pm 1{\cdot}4$ mg./100 ml. After the spawning season the blood sugar fell to a steady level of $40 \pm 1{\cdot}4$ mg./100 ml. in April and May. In June, however, there was a return of the hyperglycaemic condition, the mean value being $57 \pm 2{\cdot}4$ mg./100 ml. There was a slight, but not statistically significant, fall in July, followed by a significant decrease to $38 \pm 1{\cdot}6$ mg./100 ml. in August. From August onwards there was a steady rise, the mean reaching $51 \pm 1{\cdot}9$ mg./100 ml. in November.

From November until the onset of sexual activity the blood sugar remained in the neighbourhood of 40 mg./100 ml. In Fig. 6 the standard error of the mean of each sample has been plotted, and it can be seen that the increases in blood sugar in the hyperglycaemic periods are statistically highly significant ($P < 0.001$). The standard errors for the higher (± 2.0 to 3·5) were usually greater than those for the lower means (± 1.0 to 1·5 mg./100 ml.).

A search of the literature has revealed a few estimates of blood sugar in the frog which may be compared with those reported here. Loewit (1909) found 540 mg./100 ml. in winter frogs, while spring frogs contained 810 mg./100 ml. It is generally agreed by other authors (Bang, 1913; Lesser, 1913; Scott & Kleitman, 1921) that these values are much too high, but it is interesting to note that Loewit found a higher blood sugar in spring than in winter frogs. Bang (1913) estimated the blood sugar of freshly caught frogs (*R. temporaria*) and found it to be 40 mg./100 ml. in May, while it varied between 40 and 50 mg./100 ml. in August, values very similar to those obtained in the present investigation for the corresponding months. Lesser (1913) used composite samples of frog blood in July which on analysis by Bang's method showed 35, 40 and 36 mg. of sugar per 100 ml. of blood. Brinkmann & Van Dam (1919) found blood sugars ranging from 40 to 65 mg./100 ml. Scott & Kleitman (1921) determined the blood sugar of *R. pipiens* in February, March and April and obtained an average for their whole series of 37 mg./100 ml., there being no apparent increase during the spring, but the frogs were kept in a vivarium at 18° C., and the duration of captivity is not stated. Slome (1936) determined the blood sugar of *Xenopus laevis* when dark- and light-background adapted, and found it was significantly higher in the dark than in the light specimens (35·0 and 25·6 mg./100 ml. respectively). Similarly, after removal of the anterior lobe of the pituitary the blood sugar was reduced to 22 mg./100 ml. Zwarenstein & Bosman (1932), however, also investigated the influence of various agencies which affect the state of the melanophores on the fasting blood-sugar level of *X. laevis*. They found that exposure to white or dark backgrounds, total darkness, or removal of the eyes led to no alteration of the blood sugar. They also found that hypophysectomy, either complete or anterior lobe only, had no effect on the blood sugar, but did lead to a considerable increase in glucose tolerance. Besson (1945) carried out a similar investigation on *Rana temporaria* during the months of February, March and April. He found that the blood sugar was higher for the frogs kept on a light ground, but the difference decreased throughout his series and in April there was no background effect. Besson's mean values on a light background were as follows: February 79 mg.%, March 59 mg.% and April 36 mg.%, while on a dark background he obtained: February 48 mg.%, March 43 mg.% and April 36 mg.%. The experimental details of this work are not very clear, but it appears that the frogs were in captivity without food during the whole 3-month period of the experiment. As far as the blood sugars of the light-background adapted frogs are concerned they follow rather closely the cycle shown in Fig. 6, although Besson does not mention the occurrence of any sexual activity. The difference in blood sugar of the light- and dark-background adapted animals is

directly opposed to that found by Slome (1936) for *Xenopus laevis*. The present author made a short series of observations on the relation between blood sugar and background in March 1948. Twenty-seven frogs which had been in captivity and unfed for 2 weeks were divided into two groups, one of which was placed on a black and the other on a white background. At the time of killing all the frogs showed good adaptation to their particular background. The mean blood sugar of the frogs on the light ground was $49 \pm 3{\cdot}5$ mg.%, compared with $45 \pm 3{\cdot}6$ mg.% for those on the dark ground, the difference being without significance. It would appear, therefore, that the existence of a relation between blood sugar and background is not certainly established at present for either *X. laevis* or *Rana temporaria*.

While the previous work on the blood sugar of the frog provides little information which can be regarded as confirming the seasonal variation found in the present investigation, the extreme range of the mean values (37–62 mg.%) observed lies within that found by other workers, namely, 34–79 mg.% if the very high figures reported by Loewit are neglected.

Scott & Kleitman (1921) found that the blood sugar of the female *R. pipiens* was slightly higher than that of the male. To ascertain whether the present data provide any indication of a sex difference the monthly means for the males and females have been computed separately and are shown in Fig. 7. The splitting of the monthly samples into sex groups has, of course, reduced the sample number and there is a general tendency for the standard errors of the means given in Fig. 7 to be increased by the reduction in the number of observations in each group. In some of the samples the proportion of the sexes was unequal; for instance in June and July the samples contained only three and four females respectively, while the males in the same samples numbered eight and ten. In general, the annual cycle seen in Fig. 7 follows that already described for the undivided samples, but the detailed relationship between the sexes is of considerable interest. From October to May the female blood sugar lies slightly below that of the male in every sample, the maximum difference being 7 mg.% in November and the minimum only 1 mg.% in April and May. With the onset of the hyperglycaemia in June, however, the female blood sugar rises above that of the male and remains slightly higher until September. Again the difference is not very great, the maximum being 6 mg.% in June and falling to 2 mg.% in September. It is apparent from Fig. 7 that the differences between the sexes are not statistically significant. This is because the number of observations in each group was too small. The June, July and August observations on the one hand, and those for October, November and December on the other, may be pooled. If a statistical analysis is now made for the sex variable only, it can be shown that, in both groups, the sex difference in blood sugar is statistically significant, P being $< 0{\cdot}02$ in June, July and August, and $< 0{\cdot}001$ in October, November and December. It therefore seems probable that there was a sex difference in the 72 hr. blood-sugar level, and that there was a reversal of the relation between the sexes taking place near the end of September. The sex difference found by Scott & Kleitman (1921) for *R. pipiens* was only 7 mg.% for their whole series, and is only statistically significant if one very hypoglycaemic female (11 mg.%)

is not included. Bosman & Zwarenstein (1930) found no sex difference in the fasting blood sugar of *Xenopus laevis*. It will be advantageous to defer further discussion of the sex difference found in this work to a later section of the paper.

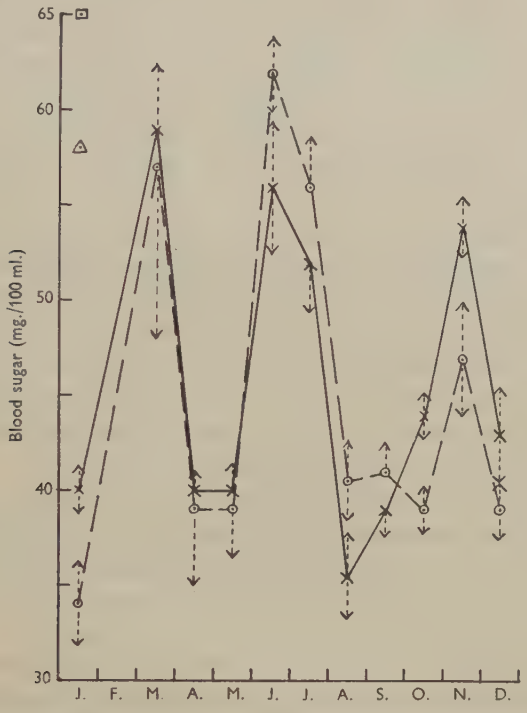

Fig. 7. Seasonal change in blood sugar of male and female *R. temporaria* during 1948, after 3 days in captivity without food. ×———×, males; ⊙- - - -⊙, females; ⊡, sexually active males Jan. 1948; △, sexually active females Jan. 1948. Vertical broken lines show the standard errors of the sample means.

Comparison of the various seasonal cycles

During the early part of the year, up to and including the spawning period, comparison of Figs. 1, 3, 5 and 6 shows that the fat body, liver weight, and liver glycogen were all decreasing. This decrease was most rapid during the breeding season itself when the blood sugar was high. It seems, then, that the hyperglycaemia accompanying sexual activity was correlated with a rapid consumption of the stored food reserves. This is not unexpected in view of the fact that not only is the metabolism of the frogs high at this time (Krogh, 1904; Dolk & Postma, 1927), but also they probably are not feeding to any appreciable extent. In connexion with this high blood-sugar level and the mobilization of liver glycogen during the breeding season it is interesting to compare the rate of sugar production by the isolated liver. Lesser (1921) perfused isolated livers of *R. esculenta* with isotonic Ringer solution. The total glucose formed during 4 hr. perfusion between August and February varied from 300 to 500 mg., but in March, April and June it increased to approximately 1080 mg., in a 4 hr. period. Fluch *et al.* (1935) also perfused isolated livers of

R. esculenta, and found this marked increase in sugar production in February only. These workers were also able to show that liver glycogenolysis was considerably reduced by previous hypophysectomy, but that such treatment did not abolish the increased sugar production observed in February. They therefore concluded that the increased glycogenolysis at this time was independent of the influence of the anterior pituitary, but might have had a connexion with spawning. The spawning season is later in the year for *R. esculenta* than for *R. temporaria*, but in view of the results of perfusion experiments given above it would seem probable that the high blood-sugar level associated with sexual activity was due to a potentiation of glycogenolysis in the liver.

In the post-spawning months of April and May the frogs were feeding actively but there was no regeneration of the fat body, or deposition of liver glycogen, while the blood sugar remained steady at 40 mg.%. This phase was succeeded by a marked change in June. As has already been shown, there was a rise in blood sugar, a simultaneous slight increase in liver weight and liver glycogen, and the fat-body weight increased rapidly. These changes in June are shown compared in Figs. 8 and 9, and it can be seen that the main feature accompanying the raised blood sugar of this period was the rapid regeneration of the fat body.

During the remainder of the year the sequence of events was different for the two sexes, and it is simpler to consider them separately. In Fig. 8 the changes in blood sugar, testis weight, fat-body weight, and liver-glycogen content are shown for the male. It can be seen that the fall in blood sugar found in August coincided very closely with the peak of the wave of spermatogenesis in the testis, as indicated by the increase in testis weight. It may also be noted that the male blood sugar attained its lowest value for the year at this time. As the wave of spermatogenesis subsided, then the blood sugar slowly rose again. It is also apparent that during spermatogenesis there was no further deposition of liver glycogen or regeneration of the fat body. When spermatogenesis was subsiding, however, there was a slight increase in the fat-body weight to its October maximum, but the main event was a large increase in liver glycogen which rose to 518 mg./100 g. of frog in October. The comparable cycle in the female for the same period is shown in Fig. 9. In this case also the blood sugar fell in August, though not as far as that of the male, and this was also accompanied by an increase in the weight of the gonads. Unlike spermatogenesis in the testis, the regeneration of the ovary continued until November, and this process was accompanied, at least until October, by a low blood-sugar level. The liver glycogen in the female did not begin to increase until October, that is, a month later than in the male, and the maximum attained was also lower in the female.

Fig. 10 shows the relationship between the blood-sugar level and gonad regeneration for the two sexes. Here the course of regeneration in the male is again indicated by the changing weight of the testis, but instead of using ovary weight in the female the ovary glycogen per 100 g. of frog has been plotted. The data for this part of the figure have been taken from the work of Bleibtreu (1911) on *R. temporaria*. The sequence of events in the male is, of course, as described above. In the female, however, the use of ovary-glycogen content instead of ovary weight shows a clearer

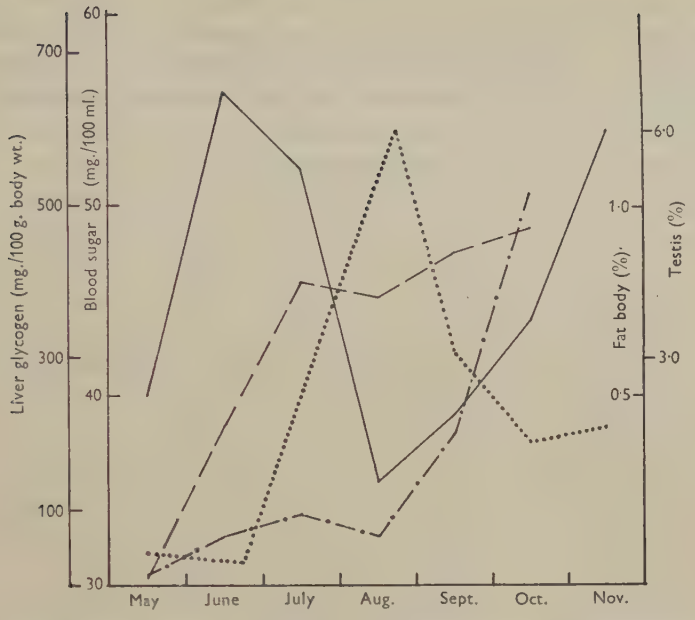

Fig. 8. Comparison of seasonal changes in blood sugar, fat-body weight, gonad weight, and liver glycogen of male *R. temporaria*. ————, blood sugar; — — —, fat body; ....., gonad; — · — · —, liver glycogen.

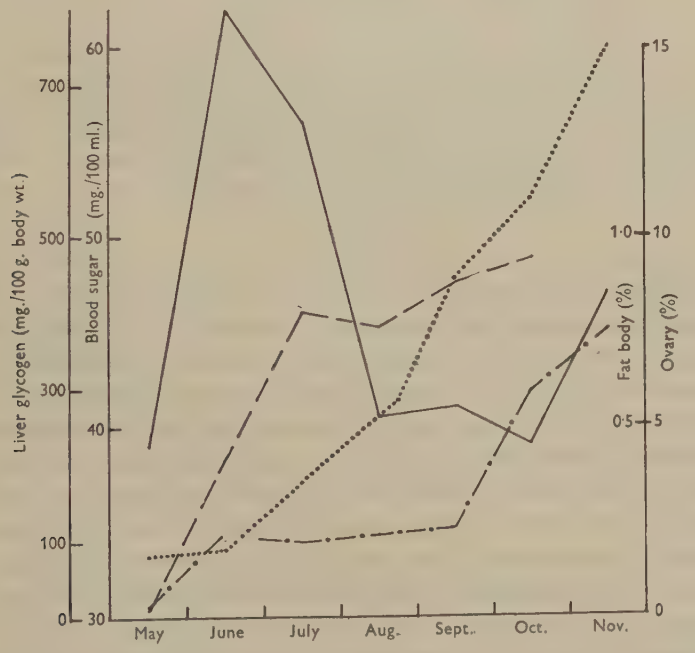

Fig. 9. Comparison of seasonal changes in blood sugar, fat-body weight, gonad weight, and liver glycogen of female *R. temporaria*. For explanation of symbols, see legend to Fig. 8.

relation between the period of low blood sugar and gonad regeneration. Thus the fall in blood sugar in August coincided with an increase in ovary glycogen, which continued through September and October. The blood sugar remained low during the whole of this period, but in November, when the blood sugar showed a slight rise, the increase in ovary glycogen was considerably reduced. A slight increase was found by Bleibtreu to continue until December.

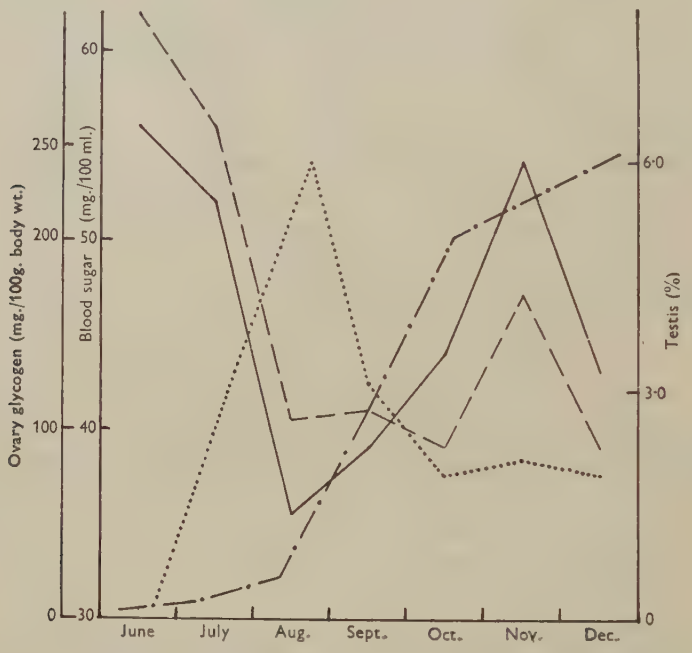

Fig. 10. Comparison of seasonal changes in blood sugar and gonad regeneration in male and female *R. temporaria*. ————, male blood sugar; — — —, female blood sugar; . . . . . , testis weight; — · — · —, glycogen content of the ovary (from Bleibtreu, 1911).

DISCUSSION

A question which arises from the results described in the preceding sections is the extent to which the observed changes may be attributed to the feeding habits of the frog. A quantitative survey of the food intake at different times of the year has not been attempted, but a note was made of the nature and amount of the stomach contents of all freshly caught frogs examined during the year. The stomach contents of the December and January samples indicated a reduction in food intake, but even in these winter months several aquatic insect larvae were found in their stomachs. These samples had been taken under water in a shallow ditch, and it must be noted that the weather of the winter 1948–9 was very mild, so that it is quite possible that in more inclement weather the frogs would have retreated into the mud at the bottom of the ditch and have ceased to feed. The fat-body cycle (Fig. 1) would suggest that even in November the food intake was inadequate for the frogs' metabolic needs, as the fat body showed a marked decline from the October level. Presumably the frog

does not feed during the spawning season, for all stomachs examined at this time were found to be empty. The liver glycogen and fat-body cycles show that the frog is compelled to draw on its stored reserves from November onwards, as both decrease steadily during the winter. This mobilization is accelerated greatly by the enhanced metabolism of the breeding season, and results in the almost complete utilization of the fat body and a marked depletion of the liver glycogen. It would seem then that the changes in the fat body and liver glycogen during the winter months may be attributed to the diminished food intake being inadequate to supply even the reduced energy requirements of the winter frog.

No change in the intensity of feeding was indicated by the stomachs examined between April and November. During this period all freshly caught frogs had stomachs well filled with a variety of insects, earthworms, slugs and caterpillars. In the first part of this period there was no increase in either the fat body or the liver glycogen, and the blood sugar remained low. This survey does not provide any indication of the fate of any food ingested in excess of that needed to supply the animal's immediate energy requirements at this time. It is possible, however, that this is a period of tissue repair after the heavy demands of the spawning season and perhaps also of growth. Examination of stomach contents has, therefore, provided no evidence to indicate that an alimentary factor might be responsible for the metabolic changes observed in summer and early autumn. Such independence of food supply is also indicated by the fact that Pflüger (1907) found little difference in glycogen content in October between frogs which had been kept without access to food since the end of August and frogs which were freshly caught.

The data provided by the present survey are not sufficient to show the physiological significance of the changes in blood sugar observed. For instance, during the period of active feeding there are two phases characterized by a low blood sugar, namely, April and May, and August and September. The first of these was accompanied by no obvious change in any of the organs examined, while the second synchronized with the regeneration of the gonads, and in the female the accumulation of glycogen in the ovary. It is suggested, therefore, that the low level of the blood sugar at these times is not due to the same endocrine balance in each case, despite the similarity of the blood-sugar values. The incidence of hyperglycaemia in June could be due to an increase in hepatic gluconeogenesis from either exogenous or endogenous material. This is indicated by the concomitant rapid storage of fat, and increase in relative liver weight and liver glycogen. When the regeneration of the gonads begins it seems that another factor enters, and the glucose formed in the liver may be utilized to provide the energy needed for gametogenesis and for storage of food reserves in the ovary. When maturation of the sex products is nearing completion the glucose formed in excess of the animal's energy requirements is apparently stored as glycogen rather than as fat, as it was in June and July. It is considered that the dependence of the incidence of glycogen storage in the liver on the completion of gonad regeneration is strongly indicated by the reversal of the sex difference in blood sugar found in September, and the earlier appearance and greater extent of the liver glycogen increase in the male. Another factor contributing

to glycogen storage in the autumn may be the decreasing mean air temperature, which will reduce the basal metabolism of a poikilothermous animal.

During the year a few estimations of blood sugar have been made at intervals other than 72 hr. after capture. These have not been either regular or very numerous, but they have indicated that the blood sugar of frogs in the field is fairly high (about 70 mg. %) from April to November. It is, therefore, possible that the seasonal changes in the 72 hr. observations may be due to different rates of fall of the blood sugar from such an initial value to a steady level of about 40 mg. %, which always seems to be attained when the frogs have been kept in captivity for longer periods (over 2 weeks). The observed variations might then be reflexions of alterations in the glucose tolerance of the frog. In this connexion reference may be made to the values for liver-glycogen content found after 4 days in captivity in November (shown by dotted lines in Figs. 4 and 5). This increase in glycogen during the additional 24 hr. of captivity was accompanied by a fall in blood sugar in both sexes. Such an observation has several interesting theoretical implications, but as it is still subject to confirmation with larger sample numbers, discussion of these will not be pursued here. In the present year the intention is to follow the changes in blood sugar and liver glycogen due to captivity and inanition continuously from the time of captivity to the attainment of a stable condition. It is hoped to obtain such data for all the periods where changes in metabolism have been indicated in this preliminary survey. If possible, glucose-tolerance estimations will also be made at the same periods. When information on points such as these is available, discussion of the physiology of seasonal changes in frog metabolism should be facilitated.

SUMMARY

1. Seasonal changes in the weights of fat body, gonads and liver, liver-glycogen content, and blood sugar have been followed by observations on monthly samples of *Rana temporaria* which had been kept in captivity for 3 days without food.

2. Three periods during which the blood sugar was high (from 54 to 62 mg./100 ml. of blood) were observed during the year: at the spawning season in March, in June and July, and in November.

3. The hyperglycaemia of June and July was accompanied by the rapid development of the fat body. This phase was succeeded by that of gonad regeneration, during which there was no further storage of fat or liver glycogen, and the blood sugar was low (about 40 mg./100 ml. of blood).

4. When gonad regeneration was nearing completion glycogen storage in the liver became the dominant feature, and the blood sugar tended to rise again.

5. A sex difference in blood sugar was observed at certain periods. From June to September the female blood sugar was higher than that of the male, but from September to January the relationship was reversed. This may be correlated with the differing rates of gonad regeneration in the two sexes.

6. During the winter months, when the food intake was reduced, the frogs utilized some of their stored fat and glycogen and the blood sugar was low (about

40 mg./100 ml.). The hyperglycaemia found at the spawning season was accompanied by the rapid depletion of the fat and glycogen stores.

7. In April and May, after the breeding season, when the frogs were feeding actively, the blood sugar was low and apparently neither fat nor glycogen was being accumulated. It is suggested that this was a period of tissue repair and possibly of growth.

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